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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
Hiroshi Miwa	)	
	)	
Application No.: 09/720,122	)	Group Art Unit: 1731
	)	Examiner: Colaianni, Michael
Filed: December 21, 2000	)	Atty. Docket: 1001-0010
	)	
For: Glass Etching Composition and	)	
Method for Frosting Using the Same	)	

DECLARATION

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Hiroshi Miwa, residing at #704, 3-3, Hara 4-chome, Sawara-ku, Fukuoka, Japan, hereby declare as follows:

1. I am well knowledgeable with the Japanese and English languages;

2. I graduated from Faculty of Pharmaceutical Science, Fukuoka University, in Fukuoka, Japan; with a Bachelor of Science (B.S.) degree of Pharmaceutical Science, in 1972;

3. I have a Ph. D. degree from Faculty of Pharmaceutical Science, Fukuoka University, in 1982:

4. Since graduation, I have been working for research on high-performance liquid chromatographic technology and published the following articles, among others:

Hiroshi Miwa: "High-performance liquid chromatographic determination of mono-, poly-and hydroxycarboxylic acids in foods and beverages as their 2-nitrophenylhydrazides", Journal of Chromatography A, 881 (2000) 365-385; and

Hiroshi Miwa: "High-performance liquid chromatographic determination of free fatty acids and esterified fatty acids in biological materials as their 2-nitrophenyl-hydrazides", Analytica Chimica Acta 465 (2002) 237-255.

5. I have also been working for the glass etching technology and am very well aware of and knowledgeable with the glass etching technology;

6. I have read the descriptions of U. S. Patent Application Serial No. 09/720,122, filed in December 21, 2000, and the Japanese counterpart;

7. I also have read all the prior art cited by the Examiner in the Office Action in connection with the above U. S. Patent Application;

8. In order to distinguish the subject matter of the U. S. Patent Application Serial No. 09/720,122 (Miwa's Invention) from the technology disclosed in the prior art cited by the Examiner and selected by the undersigned as being pertinent, in my opinion, to the subject matter as claimed in the application, I have conducted comparative studies of the Miwa's Invention with the cited prior art, i.e., U. S. Patent No. 3,616,098 to Falls (Falls '098), U. S. Patent No. 4,897,213 to Brink (Brink '213), and U. S. Patent No. 5,281,350 to Gimm et al. (Gimm et al. '350).

9. As Test #1 for the Miwa's Invention, I followed Example 10 described in the specification of the above application and prepared the etching composition.

There was added 12 grams of ammonium bifluoride to water to make the solution 100 milliliters, and 0.5 milligrams of Green Pigment #3 was added to the resulting solution. To this green solution was added 200 milligrams of propylene glycol to make the solution 300 milliliters, and 3 grams of hydroxypropyl cellulose was added. The resulting mixture was stirred to give a uniformly viscous, liquid etching composition.

The resulting etching composition was coated on one surface of a test glass piece (109 mm x 109 mm x 2 mm) with a brush and subjected to etching for 10 minutes according to the processes described in the above application. The test result is shown on the test glass piece as "Miwa", attached as "Attachment A1" to this Declaration.

10. Falls '098 discloses a method of producing an improved glare-reducing glass surface, which comprises including an undissolved inorganic salt of small crystal size, e.g., in the range of 10-50 microns in diameter and 2-10 microns in height, in the etching bath of an etching process involving cleaning the glass surface, etching in a hydrofluoric acid bath or similar acid bath containing the undissolved salt by virtue of a saturated condition of the bath, saturation of the bath being accomplished by ammonium bifluoride or other salt, and polishing the glass surface and cleaning after etching.

As Comparative Test #1, an etching composition was prepared following the process as disclosed in lines 23-27 of column 4 of Falls '098.

To a mixture of 30 grams of ammonium bifluoride crystals and 8 grams of hydrofluoric acid (based on 70% HF) with 3 grams of potassium bifluoride was added 34 grams of water, followed by adding 25 grams of diethylene glycol and mixing the ingredients to yield

an etching composition containing undissolved inorganic salts.

The resulting etching composition was then stirred well and coated with a brush on the test glass piece, followed by etching for 2 minutes according to the process as disclosed in lines 39 - 43 of column 3 of Falls '098. The test result is shown on the test glass piece as "Falls", attached as "Attachment A1" to this Declaration.

11. Brink '213 discloses a granite cleaning agent and preparation thereof and the cleaning agent comprises ammonium bifluoride: 11.2; denatured alcohol: 18.7; and water: 70.1.

For Comparative Test #2, a granite cleaning agent was prepared in accordance with the description in lines 41 - 48 of column 2 of Brink '213.

Ammonium bifluoride (11.2 grams) was dissolved in 70.1 grams of water, and 18.7 grams of denatured alcohol was mixed with the resulting solution yielding a uniform solution of the etching composition. The etching composition was then stirred well and coated with a brush on the test glass piece and then subjected to etching for 3 minutes. The test result is shown on the test glass piece as "Brink", attached as "Attachment A1" to this Declaration.

12. Gimm et al. '350 discloses an improves glass etching composition which does not contain any causative materials such as hydrofluoric acid, causing environmental pollution, which comprises a first solution of a flow modifier and ammonium bifluoride in purified glycerine, and a second solution of ammonium bifluoride and ferric chloride in purified glycerine.

Following the description in lines 10 to 41 of column 4 of Gimm et al. '350, the etching composition as Comparative Test #3 was prepared by adding 30 grams of sucrose to 100 grams of purified glycerine, heating the mixture at 90 °C in a water batch for 30 minutes, and then immediately thereafter adding 30 grams of ammonium bifluoride, followed by stirring the mixture completely and allowing the resulting mixture to cool to room temperature to give the first solution, and, separately, by heating 100 grams of purified glycerine in a 90 °C water bath for 30 minutes, adding 30 grams of ammonium bifluoride and then 10 grams of ferric chloride, stirring the resulting mixture completely and allowing the mixture to cool to room temperature, followed by filtering the residue off, to give the second solution. Then, the first and second solutions were mixed together at the rate of 1.5 (first solution) to 1.0 (second solution), i.e., 60 grams to 40 grams, and 10 grams of water was then added to the resulting mixture to give an etching composition containing crystals. The resulting etching composition was mixed well and then coated on the test glass piece with a brush and subjected to etching for 3 minutes in accordance with the process as disclosed in lines 41 and 42 of column 3 of Gimm et al. '350. The test result is shown on the test glass piece as "Gimm", attached as "Attachment A1" to this Declaration.

13. From the foregoing test results, I can conclude that the etching composition of the

Miwa's Invention is much better in performance and easier in preparation than the comparative etching compositions based on the above cited prior art.

14. Specifically, it can be noted that the comparative etching composition of the Falls '098 (Comparative Test #1) can be found hazardous because it contains toxic hydrofluoric acid and further a large amount of ammonium bifluoride while the etching composition of the Miwa's Invention does not contain any hydrofluoric acid and a very small amount of ammonium bifluoride.

Moreover, the comparative etching composition allows a portion of ammonium bifluoride and potassium bifluoride to precipitate in the solution, thereby leading to a frost with a lighter white color and irregularity in the frost.

15. It can also be noted that, as may be apparent from the comparative test result (Comparative Test #2), the comparative composition of the Brink '213 cannot or little provide a glass surface with a sufficiently high quality of frost and with a sufficiently high transparency so that the resulting frosted glass, in my opinion, cannot be used for cleaning a glass.

16. From the test result (Comparative Test #3), I find the etching composition of the Miwa's Invention highly safer and easier in preparation than the comparative etching composition of the Gimm et al. '350.

Moreover, the comparative etching composition of the Gimm et al. '350 contains ammonium bifluoride in a much more amount than that of the Miwa's Invention and it is in such a state that large amounts of sucrose and ammonium bifluoride cannot be dissolved in glycerine and their crystals are in glycerine in a suspended state because the viscosity and specific gravity of glycerine are higher. As water was added to this suspension at the rate of 10% by weight, however, the viscosity and specific gravity of glycerine are caused to become lower so that the suspension cannot stay its suspended state and allows sucrose and ammonium bifluoride to precipitate as crystals.

In addition, the comparative etching composition of the Gimm et al. '350 contains undissolved substances in a much larger amount than that of the Falls '098 and further toxic ferric chloride. It can be noted accordingly that the heating of the glycerine solution containing a large amount of ammonium bifluoride and toxic ferric chloride at elevated temperature as high as 90 °C is hazardous and dangerous to the person operating the process using the comparative etching composition. On the other hand, the etching composition of the Miwa's Invention is much safer and easier in preparation because it does not contains ammonium bifluoride so much and such toxic ferric chloride whatsoever and does not require any heating process.

Moreover, it is of my opinion that sucrose did not play any role as a gelling agent and a viscosity increasing agent.

Further, as may be apparent from the test result as per attached (Comparative Test #3),

the glass surface etched with the comparative etching composition of the Gimm et al. '350 is white in very lighter color so that the resulting frosted glass would be difficult, in my opinion, to be applicable particularly to a decoratively etched glass.

17. In summary, the comparative etching compositions of the Falls '098 and the Gimm et al. '350 contain each crystals of the ingredients while the etching composition of the Miwa's Invention does not contain any crystals so that the subject matter as claimed in this application is substantially different from those of the cited prior art. It can be noted herein that each of the comparative etching compositions provide a glass with a lighter white-colored, frosted surface as compared with a frosted glass of the Miwa's Invention.

Moreover, the comparative composition of the Brink '213 is useful for an aqueous solution for use as a cleaning agent for cleaning monuments and memorials through the use of harsh chemicals or physical removal systems, such as sand blasting so that the comparative composition is less useful as an etching composition. Therefore, the comparative composition of the Brink '213 is thoroughly different in usage from the etching composition of the Miwa's Invention.

18. For ready reference, I attach hereto three pieces of glass specimens ("Attachment A2 through A4") that were prepared in accordance with Example 1 of my U. S. patent application (Miwa's Invention). From these glass specimens, I am of the opinion that they are substantially equal in performance to the glass piece prepared above by the Miwa's Invention.

More specifically, the etching composition was prepared in the same manner as in Example 1 of this application by adding an appropriate amount of water to 12 grams of ammonium bifluoride to yield a total amount of 100 milliliters of the solution, and then adding 0.5 milligrams of Blue Pigment #1 to the resulting solution to which in turn was added 200 milliliters of propylene glycol to yield an etching composition.

Separately, there was prepared a film having a translucent portion and an opaque portion by means of photoengraving process of a photograph and design, as shown on the glass specimen and the corresponding printouts, and a silk screen print was formed, followed by masking a glass substrate with a printing ink. Further, the back side of the glass was masked, and the glass substrate was immersed in the glass etching composition for 15 minutes The glass etching composition was rinsed with tap water and the printing ink left on the glass surface was wiped out with a lacquer thinner to yield a glass having a photograph or design with the non-printed portion frosted and the printed portion left unfrosted intact.

19. For further ready reference, six sheets of photographs of frosted products ("Attachment B1 through B6") prepared in accordance with Example 10 of my U. S. patent application (Miwa's Invention) are attached to this Declaration. From these printouts, I am of the opinion that they are substantially equal in performance to the glass piece prepared above by

the Miwa's Invention.

More specifically, the etching composition was prepared in the same manner as in Example 10 of this application by adding an appropriate amount of water to 12 grams of ammonium bifluoride to yield a total amount of 100 milliliters of the solution, and then adding 0.5 milligrams of Blue Pigment #3 to the resulting solution to which in turn was added 200 milliliters of propylene glycol to make the mixture 300 milliliters. To the resulting solution was added 3 grams and 12 grams of hydroxypropyl cellulose to yield a glass etching composition having a uniform viscosity and a glass etching composition in a gel form, respectively.

Thereafter, a glass specimen was rinsed with water and water drops on the glass surface were wiped out. The glass surface was masked with an alcohol-type oil-based ink at a necessary portion thereof and then immersed in the glass etching composition or brushed therewith, followed by allowing to stand for 5 to 15 minutes. As a result, the etched glass products were obtained with the non-masked glass surface portion left unfrosted intact, as shown on the prints ("Attachment B1 through B6").

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Signed in Fukuoka, Japan, this 22 th day of January, 2004

Hiroshi Miwa

Hiroshi Miwa, Ph.D.

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Review

High-performance liquid chromatographic determination of free fatty acids and esterified fatty acids in biological materials as their 2-nitrophenylhydrazides

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Accepted 28 November 2001

Abstract

High-performance liquid chromatography (HPLC) in conjunction with direct derivatization is described for the determinations of both free fatty acids (FFAs) and esterified fatty acids (EFAs) in biological materials. The method is based on the reaction of these acids with acidic 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) with and without saponification of the samples, and there are no sample work-up steps. The derivatized FAs were extracted into *n*-hexane and separated isocratically with short retention times. These acids were consisted saturated and mono- and polyunsaturated FAs including *cis–trans* isomers and double-bond positional isomers. The analytical results showed good recovery and reproducibility using an internal standard. The method is simple, rapid and reliable and has several advantages with regard to resolution, analysis time, selectivity and sensitivity over previous methods. Thus, the present method can serve as a useful tool for routine determinations of FFAs and EFAs in various fields.

Keywords: Review; High-performance liquid chromatography; UV–visible detection; Free fatty acids; Esterified fatty acids; Serum; Total and individual platelet phospholipids; Direct derivatization; 2-Nitrophenylhydrazine hydrochloride

1. Introduction

The development of an analytical method for the routine simultaneous identification and quantification of a variety of fatty acids (FAs) is desirable for use in various fields. The determination of FAs has been developed mainly by gas chromatography (GC) of methyl esters [1–5], a technique introduced in 1952 by James and Martin [6]. As an alternative to GC, liquid chromatography (LC) has better sensitivity and selectivity. However, most FAs show no useful absorption in the visible and ultraviolet (UV) regions or no fluorescence for detection in high-performance liquid chromatography (HPLC). Therefore, several HPLC methods have been developed for the analysis of saturated and unsaturated FAs, employing pre-column derivatization techniques to increase the sensitivity and selectivity of detection [7–48].

In most of these methods, one drawback is that lengthy and cumbersome clean-up procedures, such as liquid–liquid extraction or extrelut disposable column extraction, are needed for the quantitative isolation of the FAs from the biological materials prior to the derivatization. Another drawback is that, in spite of a fairly long analysis time (40–70 min), simultaneous measurements of biologically important

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polyunsaturated FAs, such as α-linolenic acid (n = 3), γ-linolenic acid (n = 6), eicosapentaenoic acid (n = 3) (EPA), arachidonic acid (n = 6) (AA) and docosahexaenoic acid (n = 3) (DHA) could not been achieved. Therefore, the establishment of a simpler and more rapid method with complete separation capability is needed for the screening of large numbers of samples.

We have developed the utility of the reagent 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) for the derivatization of various carboxylic acids including FAs, and their separation and quantitation by HPLC in various fields [49–65]. I have already reviewed the HPLC determination of mono-, poly- and hydroxycarboxylic acids in foods and beverages as their 2-nitrophenylhydrazides [66]. The present review demonstrates the direct derivatization of free fatty acids (FFAs) in serum and esterified fatty acids (EFAs) in platelet phospholipids with 2-NPH·HCl without any pre-treatment and/or extraction steps, and the determinations of the acid hydrazides using a reversed-phase HPLC method with simple isocratic elution systems.

2. Derivatization

2.1. Reagent solutions

Phospholipid standards, L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylinositol (PI), L-α-phosphatidyl-DL-glycerol (PG), L-α-lysophosphatidylcholine, sphingomyelin, cardiolipin and triglycerides (TG) were purchased from Sigma (St. Louis, MO, USA). All FA solutions in ethanol were obtained from Yamamura Chemical Laboratories (Kyoto, Japan). 2-NPH·HCl (Tokyo Kasei Kogyo, Tokyo, Japan) solutions (20 mM) were prepared by dissolving the reagent in water, 40 mM hydrochloric acid–ethanol (3:1, v/v) and 0.3 M hydrochloric acid–ethanol (1:1, v/v). A 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (1-EDC·HCl) (Sigma, St. Louis, MO, USA) solution (0.25 M) was prepared by dissolving the reagent in a solution of pyridine (3%, v/v) in ethanol. A potassium hydroxide solution (15%, w/v) in methanol–water (4:1, v/v), a potassium hydroxide solution (10%, w/v) in methanol–water (1:1, v/v) and a potassium hydroxide (0.4 M)–ethanol (1:1, v/v) solution were prepared. All the reagent solutions were stable for at least 3 months when kept below 5 °C, and were also commercially available from Yamamura Chemical Laboratories (Kyoto, Japan). All other chemicals were of analytical-reagent grade, unless stated otherwise.

2.2. Derivatization procedure

FAs were dissolved in ethanol in various concentrations. To 50 μl of each sample solution, 100 μl of ethanol (if necessary internal standards were added), 200 μl of 2-NPH·HCl in water solution and 100 μl of 1-EDC·HCl solution were added and the mixture was heated at 60 °C for 20 min. After the addition of 50 μl of 15% (w/v) potassium hydroxide solution, the mixture was further heated at 60 °C for 15 min and then cooled. An aliquot (1–20 μl) of the resulting hydrazide mixture was injected directly into the chromatograph. The derivatized compounds were stable for at least 1 month when kept below 5 °C.

2.3. Derivatization conditions

All of the FAs, including saturated and mono- and polyunsaturated FAs react sensitively with 2-NPH·HCl using 1-EDC·HCl as a coupling agent to give acid hydrazides [49,51,52,54,55,58–60,62,64]. Those compounds gave absorption maxima at 400 nm in the acidic medium, and were detectable photometrically by monitoring at this wavelength. These derivatives also showed strong absorption in the UV region, with maximal absorption at around 230 nm, and were monitored with a UV detector. An excess of the reagents and the reaction by-products did not interfere with the HPLC analyses in the visible range, because they did not absorb visible radiation at 400 nm and were eluted before any of the FA hydrazides. The advantage of using visible detection is that the chromatograms are simpler and more selective, in spite of approximately four-fold lower sensitive than when UV detection is used [49,54].

Using this procedure, the direct derivatization of FFAs in serum without complicated isolation steps was studied. Deproteinization is one of the major problems in such a direct derivatization procedure, and it can be achieved by membrane ultrafiltration or by precipitation with an organic solvent [67,68]. Use of ethanol as

a precipitating agent for protein makes a convenient method for obtaining profiles of total organic acids in body fluids and tissue homogenates [69–71]. The ethanol content of the reaction mixture in this study was ca. 75% (v/v) [53]. In addition, the mixture was heated at 60 °C. Consequently, the protein-bound acids were deproteinized and were converted into their hydrazides [53,54,58].

During the coupling reaction, the pH of the reaction mixture is one of the principal parameters to ensure the maximum derivatization of the FAs [53,54,58]. The pH of the reaction mixture was slightly increased by the basic substances that occurred in serum samples. This led to a decrease in the yield of the acid hydrazides. The effect of the hydrochloric acid concentration of the 2-NPH·HCl solution on the peak height of margaric acid hydrazide is shown in Fig. 1. Relatively higher peaks were obtained in the concentration range of 20–50 mM hydrochloric acid without affecting the interfering peaks, and 30 mM was selected for this study [53].

FAs are present in serum principally in esterified form with triacylglycerols and glycerophospholipids. Hatsumi et al. [32] demonstrated that some FAs, such as myristic, palmitic, oleic ($n = 9$) and stearic acids, were remarkably released from PI during the commonly used process of chloroform extraction. To examine whether the FAs of these glycerolipids were derivatized, TG, PG, PC and PS (1 mg in each sample) were derivatized as described above. Under the reaction conditions, no FAs were observed in the chromatograms.

In the case of the conventional methods of analysis of EFAs in platelet phoapholipids, an extraction step is needed following saponification in order to obtain the FFAs prior to derivatization. This step might cause difficulties in the analysis of low levels and/or volatile FAs. In our HPLC method of analysis of FAs in platelet phospholipds, an acidic solution of 2-NPH·HCl, as the labeling agent, can be successfully employed for the direct derivatization of the saponified sample without the extraction step.

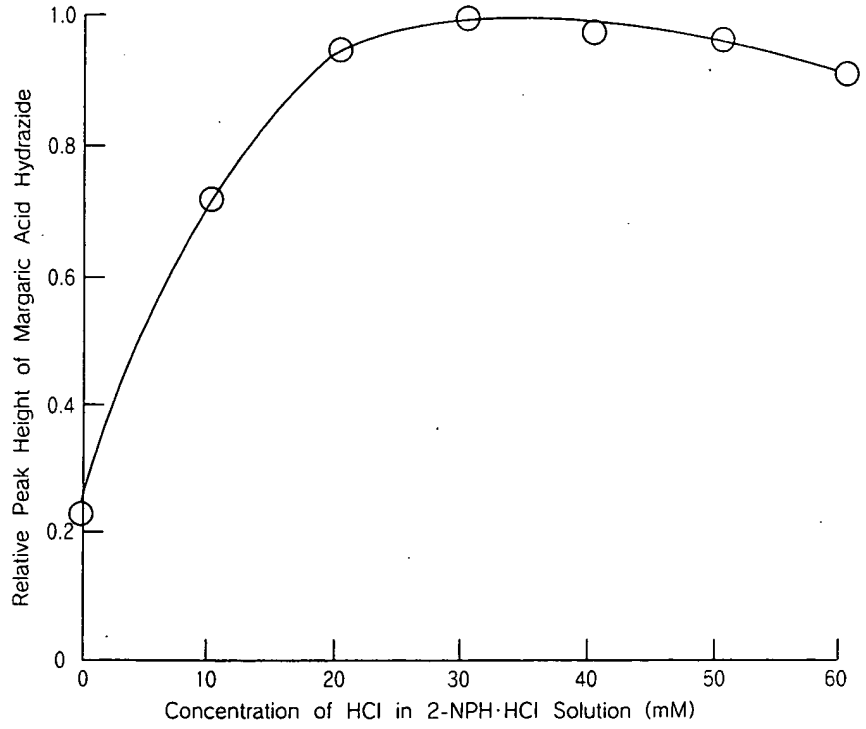

Fig. 1. Effect of the hydrochloric acid concentration in 2-NPH·HCl solution on the peak height of margaric acid: 20 nmol of margaric acid in serum was treated by assay procedure using various concentration of hydrochloric acid in 2-NPH·HCl solution.

Thus, FFAs in serum and saponified EFAs in platelet phospholipids could be directly converted into their hydrazides in high yields [53,59,60,64].

In biological materials, the amounts of FAs of interest are sometimes too low to be detected due to the sensitivity limit of the detector, and the acid hydrazides in the reaction mixture are contaminated with a manifold excess of the reagents and reaction by-products. Therefore, a purification and enrichment for the acid hydrazides is necessary. We investigated purification-enrichment by extraction of the acid hydrazides with *n*-hexane from the reaction mixture [52]. Table 1 shows that the effect of pH on the extraction efficiency of *n*-hexane was unchanged in the investigated pH region and satisfactory recovery rates were obtained. Thus, pH 6.4 phosphate buffer to 0.5 mol/l HCl ratio of 3.8:0.4 was used here to obtain higher peaks.

2.4. Assay of FFAs in human serum

To 25 μl of human serum, 25 μl of ethanol containing 2 nmol of margaric acid as internal standard, 100 μl of ethanol, 100 μl of 2-NPH·HCl in 40 mM hydrochloric acid–ethanol (3:1, v/v) solution and 100 μl of 1-EDC·HCl solution were added and the mixture was heated at 60 °C for 20 min. After the addition of 50 μl of potassium hydroxide solution (15%, w/v) in methanol–water (4:1, v/v), the mixture was further heated at 60 °C for 15 min and then cooled. To the resulting hydrazide mixture 2 ml of 1/30 M phosphate buffer (pH 6.4–0.5 M hydrochloric acid (3.8:0.4, v/v) and 1.5 ml of *n*-hexane were added. After vortexing (30 s) and centrifugation (1500 × *g*, 5 min), the *n*-hexane layer was taken and evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 50 μl of methanol and an aliquot of 2–10 μl was injected into the chromatograph.

Table 1
Effect of pH for extraction efficiency of *n*-hexane

Fatty acid	Recovery (%) (pH 6.4 buffer to 0.5 mol/l HCl)			Mean ± S.D.
	3.75:0.45 (pH 6.75)	3.80:0.40 (pH 6.82)	3.90:0.30 (pH 7.12)	
$C_{10:0}$	102.2	102.9	101.7	102.3 ± 0.5
$C_{12:0}$	101.1	99.8	99.5	100.1 ± 0.7
$C_{14:0}$	99.4	99.3	98.7	99.1 ± 0.3
$C_{14:1}$	102.7	102.5	103.0	102.7 ± 0.2
$C_{16:0}$	100.4	100.4	100.3	100.4 ± 0.05
$C_{16:1}$	99.5	99.1	99.5	99.4 ± 0.2
$C_{18:0}$	98.9	98.2	99.3	98.8 ± 0.5
$C_{18:1}$	100.6	100.0	99.4	100.0 ± 0.5
$C_{18:2}$	102.1	101.9	102.0	102.0 ± 0.1
$C_{18:3}$	103.2	103.1	102.9	103.1 ± 0.1
$C_{20:3}$	99.9	99.5	99.8	99.7 ± 0.2
$C_{20:4}$	102.2	102.6	102.2	102.3 ± 0.2
$C_{20:5}$	101.7	101.5	101.4	101.5 ± 0.1
$C_{22:6}$	103.2	103.4	102.9	103.2 ± 0.2
IS[a]	4.08	4.30	4.15	

[a] The value indicates the peak height of the internal standard (0.016× absorbance units at 230 nm).

2.5. Preparation of platelet lipids

After overnight fasting, to measure FA compositions in platelet phospholipids, blood was drawn from normal and diabetic subjects by use of a vacuumed venous aspirator containing Na_2EDTA anticoagulant (Terumo, Kyoto, Japan). The blood (ca. 7 and 20–30 ml for total and individual platelet phospholipids, respectively) was centrifuged at 225 × *g* for 6 min to obtain platelet-rich plasma (PRP). The PRP was washed twice with 0.9% sodium chloride and centrifuged at 1000 × *g* for 20 min after each washing, to obtain a platelet pellet. The lipid fraction was extracted by the method of Folch et al. [72], using a chloroform–methanol solution (2:1, v/v).

2.6. Assay of EFA in total platelet phospholipid

A total phospholipid was separated from the other lipids by thin-layer chromatography (TLC) in one dimension on a 20 cm × 5 cm × 0.25 mm silica plate (silica-gel 60 F_{254}, Merck, Darmstadt, Germany), which was developed with *n*-hexane–diethyl ether–acetic acid (65:35:1, v/v). After visualization the TLC plate with iodine vapor, the total phospholipid was scraped off from the TLC plate and extracted with 5 ml of methanol. The solvent was removed under a stream of nitrogen at room temperature, and the FAs of the phospholipids fraction were analyzed as their hydrazides.

The residue was dissolved in 200 μl of ethanol containing 50 nmol of margaric acid as the internal standard and was saponified with 100 μl of 0.4 M potassium hydroxide–ethanol (1:1, v/v) at 80 °C for 20 min. To the saponified sample, 200 μl of 2-NPH·HCl in

0.3 M hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 80 °C for 5 min. After the addition of 200 μl of potassium hydroxide solution (10%, w/v) in methanol–water (1:1, v/v), the mixture was further heated at 80 °C for 5 min and then cooled. To the resultant hydrazide mixture, 4 ml of 1/30 M phosphate buffer (pH 6.4)–0.5 M hydrochloric acid (3.8:0.4, v/v) were added. The FA hydrazides were extracted with 5 ml of *n*-hexane, and the solvent was evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 50 μl of methanol, and an aliquot (5–20 μl) was injected into the chromatograph.

2.7. Assay of EFA in individual platelet phospholipids

Individual phospholipids were separated from the other lipids by TLC in one dimension on a 20 cm × 5 cm × 0.25 mm silica plate (LK-5 with a preadsorbent area, Whatman Inc., NJ, USA), which was developed with methyl acetate–1-propanol–chloroform–methanol 0.25% (w/v) potassium chloride solution (25:35:20:10:10, v/v). After visualization the TLC plate with iodine vapor, each phospholipid class was scraped off from the TLC plate and extracted with 5 ml of methanol. The solvent was removed under a stream of nitrogen at room temperature, and the FAs of the each phospholipids class were analyzed as their hydrazides.

The residue was dissolved in 100 μl of ethanol containing 50 nmol of margaric acid as the internal standard and was saponified with 50 μl of 0.4 M potassium hydroxide–ethanol (1:1, v/v) at 80 °C for 20 min. To the saponified sample, 100 μl of 2-NPH·HCl in 0.3 M hydrochloric acid–ethanol (1:1, v/v) solution and 100 μl of 1-EDC·HCl solution were added and the mixture was heated at 80 °C for 5 min. After the addition of 100 μl of potassium hydroxide solution (10%, w/v) in methanol–water (1:1, v/v), the mixture was further heated at 80 °C for 5 min and then cooled. To the resultant hydrazide mixture, 4 ml of 1/30 M phosphate buffer (pH 6.4)–0.5 M hydrochloric acid (3.8:0.4, v/v) were added. The FA hydrazides were extracted with 5 ml of *n*-hexane, and the solvent was evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 50 μl of methanol, and an aliquot (5–20 μl) was injected into the chromatograph.

3. Chromatographic analysis

3.1. Instrumentation

Chromatographic analyses were carried out using a Shimadzu LC-6A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an on-line degasser ERC-3310 (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV–VIS detector. The detector signals were recorded on a Rikadenki multi-pen recorder (Tokyo, Japan). The column temperature was kept constant at 30–50 °C using a Shimadzu GTO-6A column oven. All columns were packed at Yamamura Chemical Labs.

3.2. HPLC conditions

The separation of 15 FA hydrazides was achieved on a YMC-FA (C_8) main column (particle size 5 μm, 250 mm × 4.6 mm i.d.) with a BBC-4-C_8 guard column (particle size 5 μm, 10 mm × 4 mm i.d.). The separation of 25 FA hydrazides was achieved on a YMC-FA (C_8) main column (particle size 5 μm, 250 mm × 6 mm i.d.) with a BBC-5-C_8 guard column (particle size 5 μm, 10 mm × 5 mm i.d.). The separation of 28 FA hydrazides was achieved on a J'sphere ODS-M 80 main column (particle size 4 μm, 250 mm × 4.6 mm i.d.) with a guard cartridge (J'sphere ODS-M 80). All eluents were maintained at about pH 4–5 by adding 0.1 M hydrochloric acid and were filtered through a fluoropore filter (pore size 0.45 μm) (Sumitomo Electric, Osaka, Japan).

3.3. HPLC separation

In the field of FA analysis by HPLC, 9-anthrylmethyl esters and phenacyl esters, including substituted phenacyl esters, are the most commonly used compounds. The separations are carried out on reversed-phase column using isocratic or gradient elution with methanol, acetonitrile and water. However, much of the work has not included certain biologically important FAs, such as EPA, AA and DHA.

The conditions for HPLC separations of saturated and mono- and polyunsaturated FA hydrazides were investigated using YMC-FA (C_8) column and different eluents comprising methanol and/or acetonitrile as the major solvent with varying amounts of water [51].

The simultaneous separation of a mixture of 15 kinds of FA hydrazides with mobile phases comprising various mixtures of methanol and water was not possible because of the long retention times and the separation of α-linolenic ($n = 3$) and EPA hydrazides, and of linoleic ($n = 6$), AA and DHA hydrazides, were difficult.

The elution volumes of the FA derivatives are affected principally by the number of carbon atoms and the number of unsaturated bonds in the FA chains [51,53,58,59]. In acetonitrile–water, unlike methanol–water, the number of unsaturated bonds seemed to be of greater importance. Also, the effect of the column temperature was important. A value of 30 °C was used here to shorten the analysis times, with good resolution. Thus, satisfactory resolution and favorable retention times (within 15 min) of the $C_{10:0}$–$C_{22:6}$ FA hydrazides were obtained in reversed-phase HPLC with isocratic elution using acetonitrile–water (85:15, v/v) at a flow rate of 1.2 ml/min, as shown in Fig. 2.

However, it is desired that the complete separation of naturally occurring polyenoic FAs including *cis–trans* isomers, which has a significant value in clinical and biological chemistry, is achieved by a simple elution mode.

Therefore, we first tried to separate 25 FA hydrazides including *cis–trans* isomers on a YMC-C8 column (250 mm × 4.6 mm i.d.) with acetonitrile–water, but could not obtain satisfactory separation. By increasing the column i.d. to 6 mm, however, we achieved improved separation, with the exception of the two pairs of acid hydrazides: linoelaidic ($C_{18:2,trans,trans}$, $n = 6$) and eicosatrienoic ($n = 3$), and elaidic ($C_{18:1,trans}$, $n = 9$) and eicosadienoic ($n = 6$). In order to resolve these adjacent peaks, we tried the use of a different elution system, comprising acetonitrile, methanol and water in the various proportions [58,59]. Acetonitrile and methanol have different effects on the two parameters for the elution volumes of FA hydrazides [51,53,58,59]. The column temperature was kept constant at 35 °C. Fig. 3 shows a typical separation of the 25 FA hydrazides by HPLC analysis with acetonitrile–methanol–water (75:11:14, v/v) as the eluent at a flow rate of 1.2 ml/min.

A number of studies have stimulated the belief that $n = 3$ FAs may have an essential function in certain animal cells [73–77]. However, resolution of

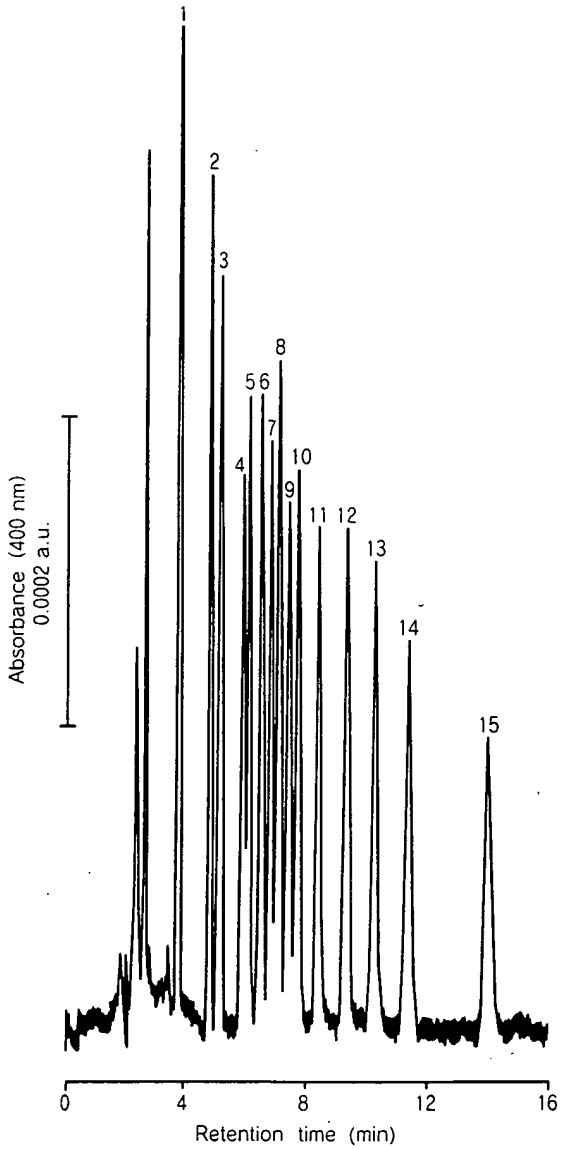

Fig. 2. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 15 fatty acids obtained with visible detection. Peaks: (1) capric ($C_{10:0}$); (2) lauric ($C_{12:0}$); (3) myristoleic ($C_{14:1}$, $n = 5$); (4) eicosapentaenoic ($C_{20:5}$, $n = 3$); (5) α-linolenic ($C_{18:3}$, $n = 3$); (6) myristic ($C_{14:0}$); (7) docosahexaenoic ($C_{22:6}$, $n = 3$); (8) palmitoleic ($C_{16:1}$, $n = 7$); (9) arachidonic ($C_{20:4}$, $n = 6$); (10) linoleic ($C_{18:2,cis,cis}$, $n = 6$); (11) eicosatrienoic ($C_{20:3}$, $n = 3$) and dihomo-γ-linolenic ($C_{20:3}$, $n = 6$); (12) palmitic ($C_{16:0}$); (13) oleic ($C_{18:1,cis}$, $n = 9$); (14) margaric ($C_{17:0}$) (internal standard); (15) stearic ($C_{18:0}$) acid hydrazide. Each peak corresponds to 10 pmol.

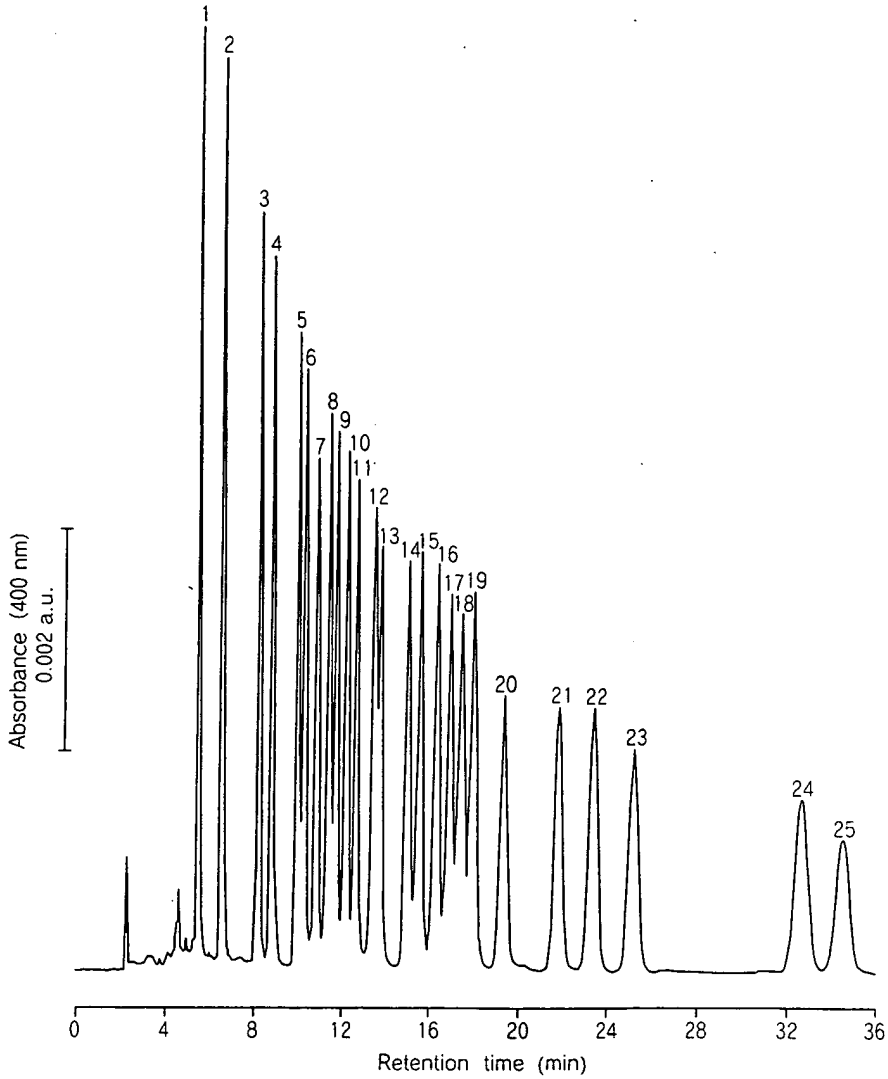

Fig. 3. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 25 fatty acids obtained with visible detection. Peaks: (1) caprylic ($C_{8:0}$); (2) capric ($C_{10:0}$); (3) lauric ($C_{12:0}$); (4) myristoleic ($C_{14:1}$, $n = 5$); (5) eicosapentaenoic ($C_{20:5}$, $n = 3$); (6) α-linolenic ($C_{18:3}$, $n = 3$); (7) myristic ($C_{14:0}$); (8) docosahexaenoic ($C_{22:6}$, $n = 3$); (9) palmitoleic ($C_{16:1}$, $n = 7$); (10) arachidonic ($C_{20:4}$, $n = 6$); (11) linoleic ($C_{18:2,cis,cis}$, $n = 6$); (12) linoelaidic ($C_{18:2,trans,trans}$, $n = 6$); (13) eicosatrienoic ($C_{20:3}$, $n = 3$) and dihomo-γ-linolenic ($C_{20:3}$, $n = 6$); (14) palmitic ($C_{16:0}$); (15) docosatetraenoic ($C_{22:4}$, $n = 6$); (16) oleic ($C_{18:1,cis}$, $n = 9$); (17) elaidic ($C_{18:1,trans}$, $n = 9$); (18) eicosadienoic ($C_{20:2}$, $n = 6$); (19) margaric ($C_{17:0}$) (internal standard); (20) docosatrienoic ($C_{22:3}$, $n = 3$); (21) stearic ($C_{18:0}$); (22) eicosenoic ($C_{20:1}$, $n = 9$); (23) docosadienoic ($C_{22:2}$, $n = 6$); (24) arachidic ($C_{20:0}$); (25) erucic ($C_{22:1}$, $n = 9$) acid hydrazide. Each peak corresponds to 150 pmol.

double-bond positional isomers, such as γ-linolenic ($n = 6$) and α-linolenic ($n = 3$) acids and $n = 9$, 12, and 15 eicosenoic acids, is not always achieved.

Therefore, the conditions for HPLC separations of FA hydrazides including double-bond positional isomers were investigated using J'sphere ODS-M 80 column with different isocratic eluents consisting of acetonitrile and water in various proportions [62,64].

Our previous works [58,59,62,64] showed that acetonitrile has a significant effect on the two factors

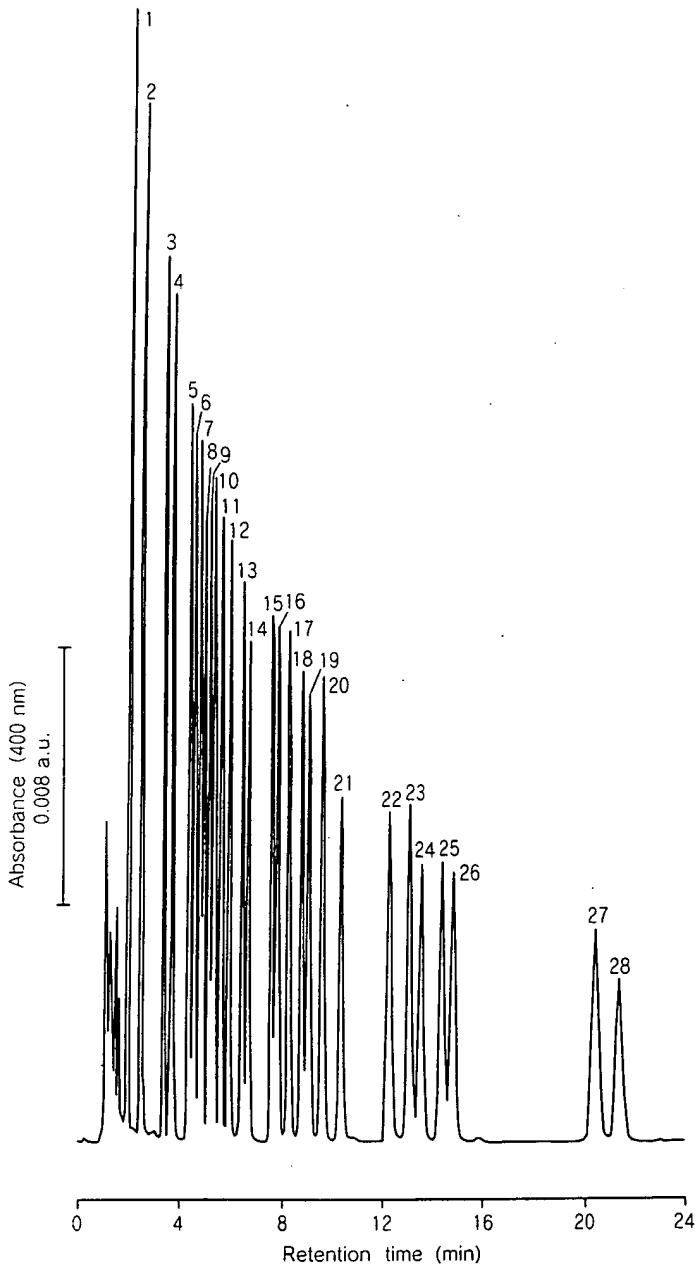

Fig. 4. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 28 fatty acids obtained with visible detection. Peaks: (1) caprylic ($C_{8:0}$); (2) capric ($C_{10:0}$); (3) lauric ($C_{12:0}$); (4) myristoleic ($C_{14:1}$, $n = 5$); (5) eicosapentaenoic ($C_{20:5}$, $n = 3$); (6) α-linolenic ($C_{18:3}$, $n = 3$); (7) γ-linolenic ($C_{18:3}$, $n = 6$); (8) myristic ($C_{14:0}$); (9) docosahexaenoic ($C_{22:6}$, $n = 3$); (10) palmitoleic ($C_{16:1}$, $n = 7$); (11) arachidonic ($C_{20:4}$, $n = 6$); (12) linoleic ($C_{18:2,cis,cis}$, $n = 6$); (13) linoelaidic ($C_{18:2,trans,trans}$, $n = 6$); (14) eicosatrienoic ($C_{20:3}$, $n = 3$) and dihomo-γ-linolenic ($C_{20:3}$, $n = 6$); (15) palmitic ($C_{16:0}$); (16) docosatetraenoic ($C_{22:4}$, $n = 6$); (17) oleic ($C_{18:1,cis}$, $n = 9$); (18) elaidic ($C_{18:1,trans}$, $n = 9$); (19) eicosadienoic ($C_{20:2}$, $n = 6$); (20) margaric ($C_{17:0}$) (internal standard); (21) docosatrienoic ($C_{22:3}$, $n = 3$); (22) stearic ($C_{18:0}$); (23) eicosenoic ($C_{20:1}$, $n = 9$); (24) eicosenoic ($C_{20:1}$, $n = 12$); (25) docosadienoic ($C_{22:2}$, $n = 6$); (26) eicosenoic ($C_{20:1}$, $n = 15$); (27) arachidic ($C_{20:0}$); (28) erucic ($C_{22:1}$, $n = 9$) acid hydrazide. Each peak corresponds to 150 pmol.

affecting the elution volumes of FA hydrazides. By increasing the proportion of acetonitrile in the eluent, γ-linolenic ($n = 6$) and myristic acid hydrazides were resolved, as were DHA and palmitoleic acid ($n = 7$) hydrazides. This change, however, also resulted in decreased resolutions of palmitic and docosatetraenoic acid ($n = 6$) hydrazides and of oleic ($n = 9$) and elaidic acid ($C_{18:1,trans}$, $n = 9$) hydrazides. Column temperature also has a significant effect on resolution. Increasing the column temperature from 30 to 50 °C leads to inversion in selectivity. Therefore, increasing column temperature caused greater resolutions of latter two critical pairs but loss of resolutions of former two critical pairs. For this study, column temperature was set at 50 °C to shorten analysis time and yet achieve good resolution. However, resolution of eicosatrienoic ($n = 3$) and dihomo-γ-linolenic acid ($n = 6$) hydrazides could not be achieved. Fig. 4 shows a chromatogram of a mixture of saturated and mono- and polyunsaturated FA hydrazides ($C_{8:0}$–$C_{22:6}$), including *cis–trans* isomers and double-bond positional isomers, obtained by using acetonitrile–water (86:14, v/v) as the eluent at a flow rate of 2.0 ml/min. Separation of the 28 FA hydrazides is performed within only 22 min by elution in the isocratic mode, which is a distinct advantage over gradient elution techniques. The same separation has hitherto been achieved only with capillary GC.

The retention times increased with increasing chain length for the FA hydrazides and inversely with the degree of unsaturation for the unsaturated FA hydrazides. The *trans* isomers were eluted after the corresponding *cis* isomers [58,59,62,64]. These chromatographic behaviors of the hydrazine derivatives agree with those of other FA derivatives.

3.4. Column lifetime

In order to serve as a useful tool for routine analysis of FFAs and EFAs, the column lifetime is one of the major problems in the HPLC analysis. The HPLC conditions employed achieved a simple isocratic separation of each acid group and the eluents comprising methanol and/or acetonitrile as the major solvent with small amounts of water did not contain buffered solution. In addition, the purification-enrichment step in the assay procedures produced only the acid hydrazides and the excess of reagents on the chromatograms. Even after more than 500 analyses, therefore, each column has shown no loss of separation capability.

4. Quantitative analysis

4.1. Calculation

Calibration curves were constructed by derivatizing increasing amounts of FAs in the presence of margaric acid as the internal standard and analyzing as described above. The calibration test was replicated five times. From the chromatograms obtained, the relationships between the peak-height ratios of the acid hydrazides to that of the internal standard and the concentrations of the acids were calculated by the least-squares method. Previous works [51,52,58,59,62] demonstrated that the calibration curves of individual FAs were linear over a wide concentration range with good correlation coefficients (0.999–1.000). The limits of detection, based on a signal-to-noise ratio of 2, were 500 fmol–4 pmol per injection. Thus, the amounts of individual FAs in samples were calculated by the internal standard method.

4.2. Recovery and precision

The recovery and reproducibility for the assay of FFAs in serum were investigated six times by adding a known mixture of 14 FAs (palmitic, stearic, oleic ($n = 9$) and α-linolenic ($n = 3$) acids 1 nmol each, others 0.3 nmol) to 25 μl of human serum. The recoveries of the FAs were in the range 98.3–103.4% and the coefficients of variation were in the range 0.7–3.1%. The intra-assay precision was evaluated by assaying six times the same human serum sample. The inter-assay precision was determined by assaying spiked human serum on different days over 1 week ($n = 6$). The intra- and inter-assay coefficients of variation were less than 2.7 and 3.5%, respectively. These results indicate that the present method has a satisfactory precision in analyzing FA levels in serum [54].

In order to examine the recovery and reproducibility for the assay of EFAs in total and individual platelet phospholipids known amounts (10 and 40 nmol) of mixtures of FAs were added to PC (0.05 mg). Each aliquot was analyzed by three separate measurements.

Table 2
Analytical recovery of fatty acids added to L-α-phosphatidylcholine

Fatty acid	Added (nmol)	Found (nmol)[a]	Recovery (%)[a]	CV (%)	Added (nmol)	Found (nmol)[a]	Recovery (%)[a]	CV (%)
$C_{10:0}$	10	9.84 ± 0.08	98.4 ± 0.8	0.8	40	39.47 ± 0.49	98.7 ± 1.2	1.2
$C_{12:0}$	10	9.99 ± 0.14	99.9 ± 1.4	1.4	40	39.95 ± 0.35	99.9 ± 0.8	0.8
$C_{14:0}$	10	9.98 ± 0.19	99.8 ± 1.9	1.9	40	39.65 ± 0.16	99.1 ± 0.4	0.4
$C_{14:1}$	10	10.03 ± 0.14	100.3 ± 1.4	1.4	40	39.27 ± 0.40	98.2 ± 1.0	1.0
$C_{16:0}$	10	10.00 ± 0.17	100.0 ± 1.7	1.7	40	39.88 ± 0.41	99.7 ± 1.0	1.0
$C_{16:1}$	10	9.82 ± 0.17	98.2 ± 1.7	1.7	40	40.68 ± 0.85	101.7 ± 2.1	2.1
$C_{18:0}$	10	10.04 ± 0.20	100.4 ± 2.0	2.0	40	39.52 ± 0.39	98.8 ± 1.0	1.0
$C_{18:1}$	10	10.06 ± 0.11	100.6 ± 1.1	1.1	40	39.89 ± 0.16	99.7 ± 0.4	0.4
$C_{18:2}$	10	10.02 ± 0.15	100.2 ± 1.5	1.5	40	40.24 ± 0.25	100.6 ± 0.6	0.6
$C_{18:3}$	10	10.04 ± 0.15	100.4 ± 1.5	1.5	40	39.54 ± 0.20	98.9 ± 0.5	0.5
$C_{20:3}$	10	9.83 ± 0.17	98.3 ± 1.7	1.7	40	41.05 ± 0.64	102.6 ± 1.6	1.6
$C_{20:4}$	10	10.14 ± 0.10	101.4 ± 1.0	1.0	40	40.88 ± 0.77	102.2 ± 1.9	1.9
$C_{20:5}$	10	9.87 ± 0.26	98.7 ± 2.6	2.6	40	40.27 ± 0.73	100.7 ± 1.8	1.8
$C_{22:6}$	10	9.94 ± 0.12	99.4 ± 1.2	1.2	40	39.24 ± 0.89	98.1 ± 2.2	2.2

[a] Mean ± S.D. ($n = 3$).

Table 2 shows the recoveries of each FA; the range of 98.1–102.6% is sufficient for practical applications. The intra-assay precision was evaluated by assaying six times the same PC. The inter-assay precision was determined by analyzing spiked PC on different days over 1 week ($n = 6$). Table 3 shows that the present method has a satisfactory precision, the intra- and inter-assay coefficients of variation ranged from 1.0 to 2.5% and from 1.4 to 3.2%, respectively. These results indicate that the present method can be used for quantitative analysis of EFAs incorporated into platelet phospholipids.

Platelet lipids extracted by the method of Folch et al. [72] were isolated by developing on the TLC plate to obtain platelet phospholipids. In order to evaluate the isolation procedure, a standard solution containing known amounts of PC (5 mg/ml in chloroform solution) was made as the model of platelet phospholipids. From this standard solution, six aliquots of 100 μl were developed on the TLC plates and analyzed as described in the assay procedures. Another six aliquots of 100 μl were analyzed directly. Table 4 lists the FA compositions obtained by the two methods. The relative recovery from TLC ranged from 98.4 to 101.0%. It can be seen that the values are not affected by the

Table 3
Precision of the direct method for determination of fatty acids in L-α-phosphatidylcholine

Fatty acid	Intra-assay ($n = 6$)		Inter-assay ($n = 6$)	
	Mean ± S.D. (nmol/mg)	CV (%)	Mean ± S.D. (nmol/mg)	CV (%)
$C_{12:0}$	10.51 ± 0.26	2.5	11.12 ± 0.23	2.1
$C_{14:0}$	15.97 ± 0.22	1.4	14.82 ± 0.24	1.6
$C_{16:0}$	931.73 ± 21.43	2.3	908.42 ± 16.35	1.8
$C_{16:1}$	26.83 ± 0.40	1.5	27.51 ± 0.63	2.3
$C_{18:0}$	403.24 ± 7.66	1.9	421.38 ± 5.90	1.4
$C_{18:1}$	767.90 ± 7.68	1.0	785.10 ± 18.06	2.3
$C_{18:2}$	372.62 ± 4.47	1.2	351.47 ± 7.38	2.1
$C_{20:3}$	15.33 ± 0.37	2.4	16.43 ± 0.46	2.8
$C_{20:4}$	94.94 ± 1.71	1.8	90.89 ± 2.45	2.7
$C_{22:6}$	20.19 ± 0.42	2.1	21.95 ± 0.70	3.2

Table 4
Fatty acid compositions of L-α-phosphatidylcholine analyzed with and without isolation by TLC

Fatty acid	Fatty acid composition (mean ± S.D., $n = 6$) (mol%)	
	With isolation	Without isolation
$C_{14:0}$	0.21 ± 0.01	0.21 ± 0.01
$C_{16:0}$	36.75 ± 0.25	36.67 ± 0.20
$C_{16:1}$	0.84 ± 0.03	0.83 ± 0.03
$C_{18:0}$	13.38 ± 0.15	13.25 ± 0.13
$C_{18:1,cis}$	29.55 ± 0.24	29.71 ± 0.08
$C_{18:2,cis,cis}$	14.16 ± 0.08	14.21 ± 0.13
$C_{20:4}$	3.08 ± 0.05	3.11 ± 0.10
$C_{22:5}$	1.39 ± 0.04	1.38 ± 0.05
$C_{22:6}$	0.64 ± 0.02	0.63 ± 0.02

isolation procedure. The intra-assay precision of the proposed method, calculated from six repeated analyses of identical platelet lipid samples, ranged from 0.3 to 4.7%. The present method has a satisfactory precision in analyzing the FA compositions of platelet phospholipids, while reducing the overall analysis time and cutting the requirement for skilled operation that applies in GC analysis [59,60].

In order to evaluate the separation procedure, a standard solution containing known amounts of PC, PE, PS and PI (1 mg/ml of each in chloroform solution) was made as the model of individual phospholipids into platelet. From this standard solution, six aliquots of 200 μl were developed on the TLC plates and analyzed as described in the assay procedures. Another six aliquots of 200 μl were analyzed directly. The differences of the fatty acid compositions incorporated into the four phospholipids were below 2.1% between with and without TLC isolation procedure. The relative recoveries of the individual phospholipids were in the range 97.2–103.8% and the coefficients of variation were in the range 0.4–3.2%. The present method has a satisfactory precision in analyzing the FA compositions of individual platelet phospholipids [64].

5. Application

5.1. Determination of FFAs in human serum

FFAs in blood, derived mainly from triacylglycerols in adipose tissues by the action of hormone-sensitive lipase or from lipoprotein fractions by the action of lipoprotein lipase, play a central role in energy metabolism. The monitoring of individual FA levels in serum or plasma is known to be useful in the management of patients with several diseases, such as diabetes mellitus [78], thyremphraxis and hepatic dysfunction [79].

To confirm the clinical utility, serum samples from 16 fasting normal subjects (10 men and 6 women) and 21 fasting patients with non-insulin-dependent diabetes mellitus (NIDDM) (8 men and 13 women) were analyzed. The FA profiles of serum samples from a normal control and a diabetic subject are shown in Figs. 5 and 6, respectively. The chromatograms monitored by visible absorbance show a very clean background, and thus the FAs in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards.

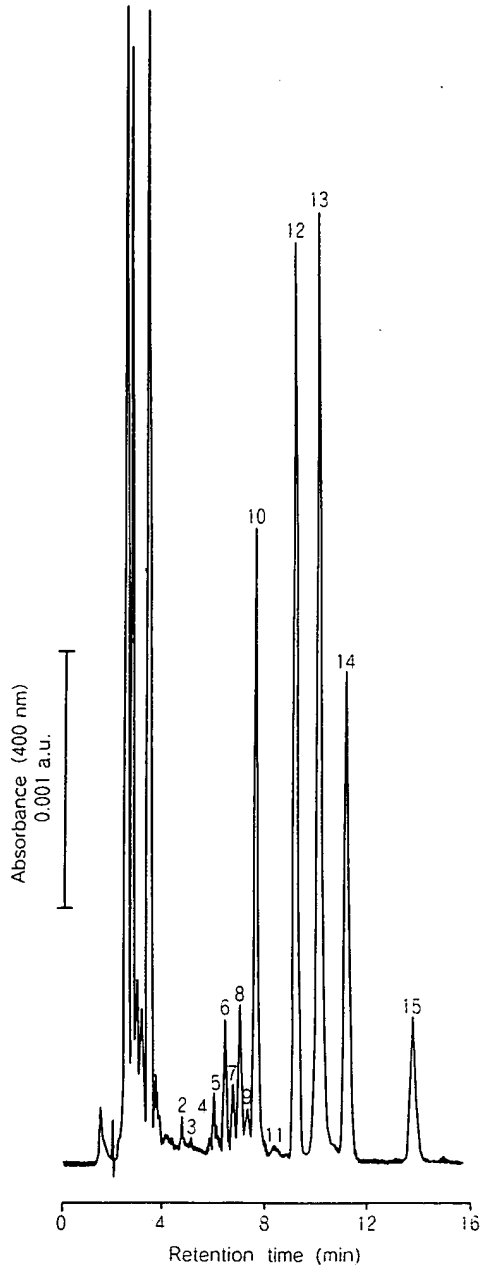

Fig. 5. FFA profile of serum obtained from a normal subject. Each peak number corresponds to that in Fig. 2.

The total concentrations and the compositions of FFAs in the sera from normal subjects and patients

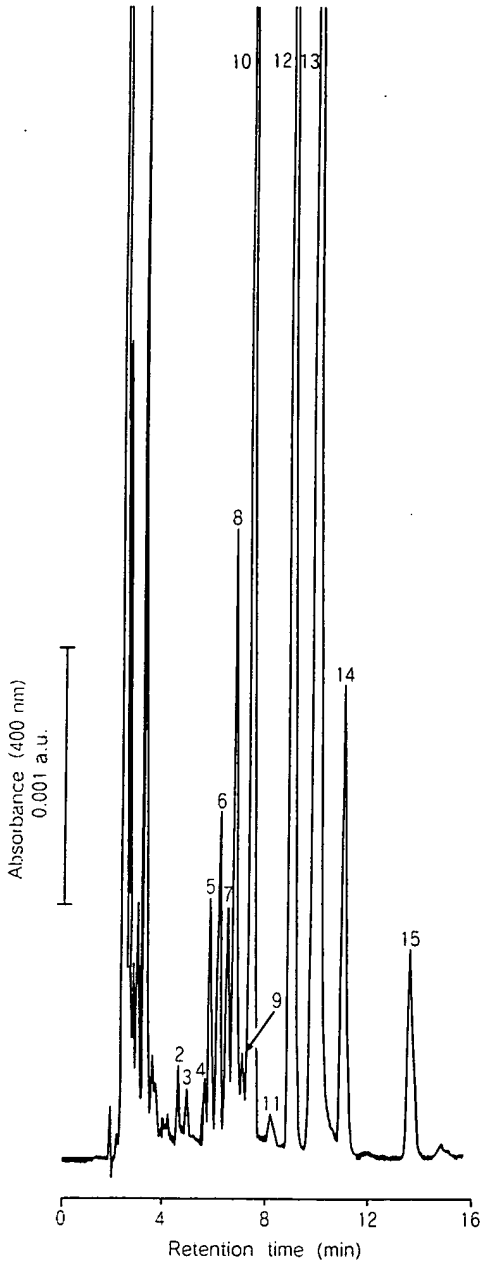

Fig. 6. FFA profile of serum obtained from a patient with diabetes mellitus. Each peak number corresponds to that in Fig. 2.

with NIDDM are listed in Table 5. The data for the individual FFAs in normal sera were in good agreement with those in other reports [20,24,52]. The mean values for the total concentration of FFAs was more than 2.5-fold higher in the patients with NIDDM than in the normal subjects. In such patients, the compositions of stearic and AA were markedly decreased ($P < 0.01$ and 0.005, respectively), whereas α-linolenic acid ($n = 3$) was significantly increased ($P < 0.01$). There was a tendency for the compositions of palmitoleic ($n = 7$), EPA and DHA in the patients to increase, but without reaching statistical significance. The ratio of unsaturated FAs to saturated FAs did not differ significantly between the two groups. Further studies are necessary to investigate whether such differences are of clinical significance and are correlated with other parameters, such as blood sugar, glycosylated hemoglobin and ketone bodies.

Table 5
FFA compositions of serum specimens from normal and diabetic subjects[a]

Fatty acid	FFA composition (mol%)	
	Normal ($n = 16$)	Diabetic ($n = 21$)
$C_{12:0}$	0.81 ± 0.48	0.69 ± 0.24
$C_{14:0}$	3.32 ± 1.31	3.14 ± 0.51
$C_{14:1}$	0.36 ± 0.13	0.42 ± 0.27
$C_{16:0}$	27.13 ± 2.19	27.52 ± 2.18
$C_{16:1}$	3.49 ± 0.81	4.29 ± 1.53
$C_{18:0}$	7.91 ± 1.58	6.38 ± 1.48*
$C_{18:1}$	30.93 ± 3.29	29.89 ± 1.96
$C_{18:2}$	19.18 ± 1.83	20.16 ± 2.52
$C_{18:3}$	1.77 ± 0.30	2.13 ± 0.45*
$C_{20:3}$	0.36 ± 0.10	0.39 ± 0.16
$C_{20:4}$	1.79 ± 0.51	1.34 ± 0.22**
$C_{20:5}$	0.66 ± 0.33	0.87 ± 0.30
$C_{22:6}$	2.29 ± 1.07	2.77 ± 0.60
Unsaturated fatty acids	60.82 ± 4.00	62.27 ± 3.44
Total concentration (nmol/ml)	321.26 ± 95.41	813.07 ± 280.03***

[a] Data are expressed as the mean ± S.D. The significant difference between the two groups was assessed using the Student's *t*-test.
* $P < 0.01$.
** $P < 0.05$.
*** $P < 0.001$.

5.2. Determination of EFAs in total platelet phospholipid

It is well known that alterations of the tissue FA composition are found in macro- and microvascular diseases, reflecting a disturbed FA metabolism. For

example, the decrease in the ratio of EPA to AA in blood lipids is one of the risk factors in coronary heart disease [80].

To confirm the practical utility, EFA compositions of platelet phospholipid samples from 10 fasting normal subjects (age range 28–52 years) and 11 fasting patients with NIDDM (age range 18–76 years) were analyzed. The EFA profiles of platelet phospholipid samples from a normal control and a diabetic subject are shown in Figs. 7 and 8, respectively. There were no interfering peaks other than FA hydrazides on the chromatograms, which were easily identified by comparison of their retention times with those of standards. In Figs. 7 and 8 the peak X is unknown, but this FA is probably identical with docosapentaenoic acid ($n = 3$), on the basis of reference chromatograms of PUFA-1 and PUFA-2 obtained from Supelco (Bellefonte, PA, USA). In this study, 14 FAs were detected in human platelet phospholipids.

The mean values for the relative EFA compositions of platelet phospholipids obtained from normal subjects and patients with NIDDM are listed in Table 6. The EPA composition was significantly higher in the patients (2.61 ± 0.30 mol%) than in the normal subjects (1.59 ± 0.30 mol%). The proportion of DHA was considerably higher in the patients than in the normal subjects, but without reaching statistical significance.

Table 6
Fatty acid compositions of platelet phospholipids from normal and diabetic subjects

Fatty acid	Fatty acid composition (mean ± S.E.) (mol%)	
	Normal ($n = 10$)	Diabetic ($n = 11$)
$C_{12:0}$	0.06 ± 0.01	0.06 ± 0.01
$C_{14:0}$	0.67 ± 0.21	0.63 ± 0.12
$C_{16:0}$	27.26 ± 0.52	28.39 ± 1.03
$C_{16:1}$	0.22 ± 0.03	0.23 ± 0.03
$C_{18:0}$	16.13 ± 0.62	13.53 ± 1.31
$C_{18:1,cis}$	10.39 ± 0.54	9.00 ± 0.91
$C_{18:2,cis,cis}$	20.37 ± 1.43	20.40 ± 2.11
$C_{18:3}$	0.12 ± 0.02	0.08 ± 0.01
$C_{20:1}$	0.22 ± 0.03	0.21 ± 0.03
$C_{20:2}$	0.25 ± 0.04	0.10 ± 0.02
$C_{20:4}$	14.28 ± 1.02	14.16 ± 0.83
$C_{20:5}$	1.59 ± 0.30	2.61 ± 0.30*
$C_{22:6}$	2.25 ± 0.13	1.83 ± 0.15
$C_{22:6}$	6.19 ± 0.37	8.77 ± 0.92

* $P < 0.05$.

There was a tendency for the proportion of stearic acid and oleic acid ($n = 9$) in the patients to decrease. One possible explanation for these observations is that the diabetic patients have undergone treatment with an EPA-rich diet, which is thereafter obviously incorporated into the platelet membranes. These findings are in keeping with observations by Siess et al. [81], who found marked changes in platelet FAs caused by dietary supplements.

Increased platelet aggregation, which is probably mediated by AA metabolites formed via both the cyclooxygenase and lipoxygenase pathways, has been described in diabetes mellitus. In such patients, however, the AA composition has been found both to increase [82] and to decrease [83] compared with normal subjects. In this study, there was no significant difference in the AA composition between the two groups. A likely explanation for the discrepancy is related to patient selection, in particular the duration of diabetes and the existence of complications, as well as the dietary factors [83].

5.3. Determination of EFAs in individual platelet phospholipids

The phospholipids vary widely between subclasses and different organs [84–86] and variations can also be induced under certain pathological conditions [87,88].

To confirm the practical utility of the present method in the assay of biological materials, we applied it to the quantitation of EFAs incorporated into each major phospholipid class (PC, PE, PS and PI) of platelets obtained from 10 fasting NIDDM patients (age range 32–66 years). In the TLC, glycolipids and neutral lipids including FAs, which have an R_F substantially greater than the farthest migrating class of phospholipid, such as PG, did not interfere with the phospholipid separation.

The FA profile of PE in platelet obtained from a patient with NIDDM after EPA-E administration is shown in Fig. 9. In the chromatogram monitored by visible absorbance, a few unknown peaks appeared, but the FAs in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards. These results demonstrated that the TLC–HPLC method described here reduced overall analysis time and decreased the sample volume required.

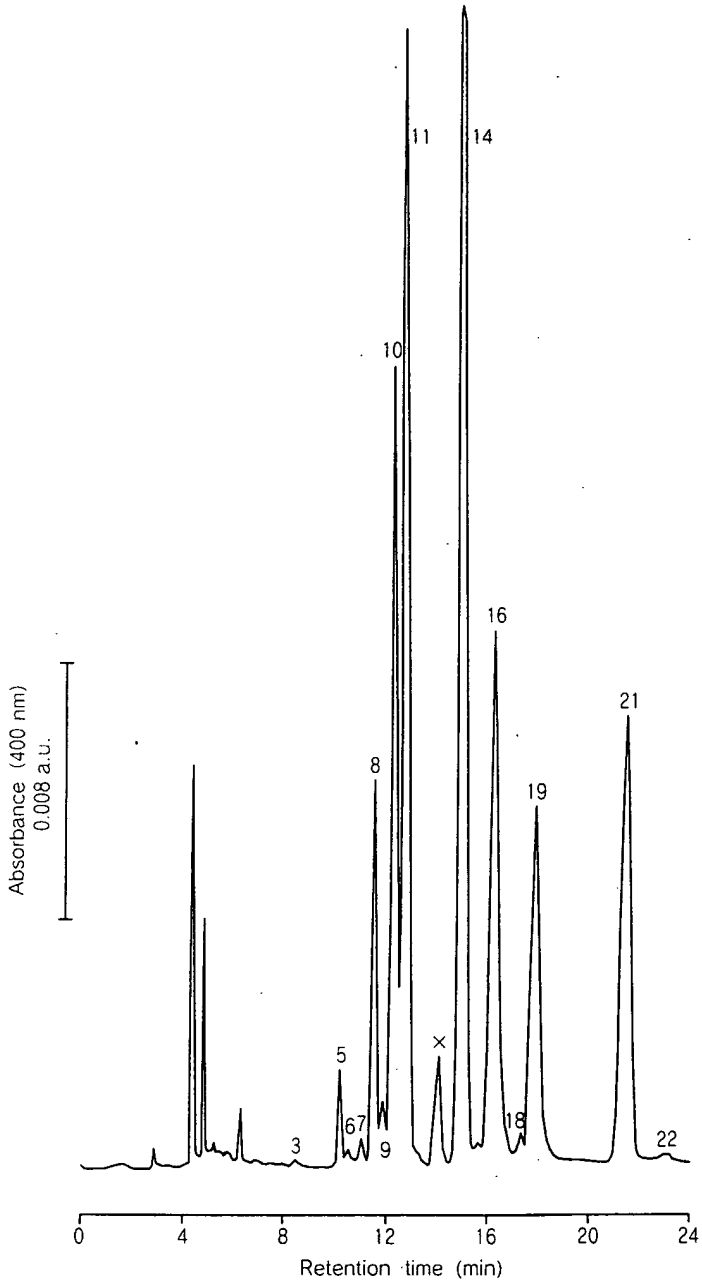

Fig. 7. EFA profile of platelet phospholipids obtained from a normal subject. Each peak number corresponds to that in Fig. 3.

Table 7 listed the mean values for the relative FA compositions of the four major phospholipids in platelets obtained from diabetic patients before and after EPA-E administration (Epadel, Mochida Pharmaceuticals, Tokyo, Japan, administration order, 1.8 g per day) for 13–22 weeks (mean 17 weeks). The FA compositions showed different patterns, with a significant increase of EPA in all phospholipid classes.

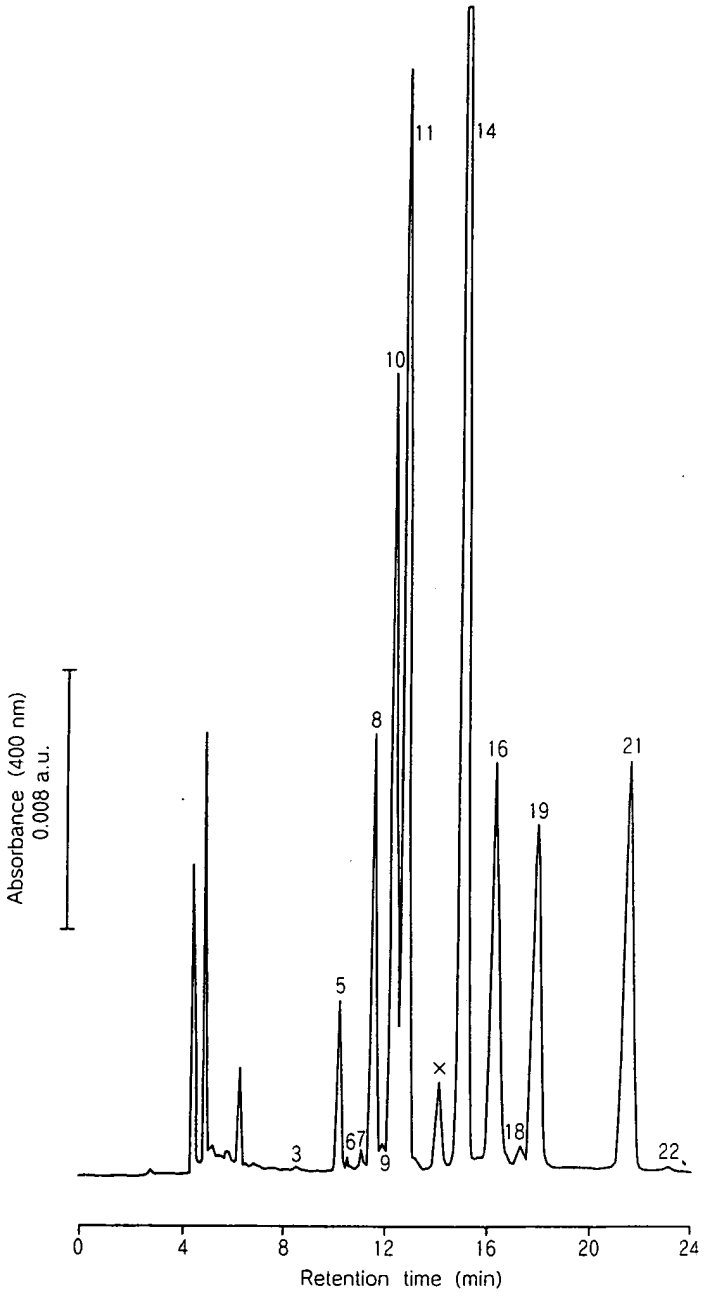

Fig. 8. EFA profile of platelet phospholipids obtained from a patient with diabetes mellitus. Each peak number corresponds to that in Fig. 3.

The EPA composition of platelet PC in the patients increased significantly with administration of EPA-E, while the AA composition decreased markedly. In platelet PE the drastically increased incorporation of EPA was associated with a significant decrease in the propositions of palmitic, stearic and arachidic acids. The proportion of linoleic acid ($n = 6$) was significantly increased only in PE. These results showed

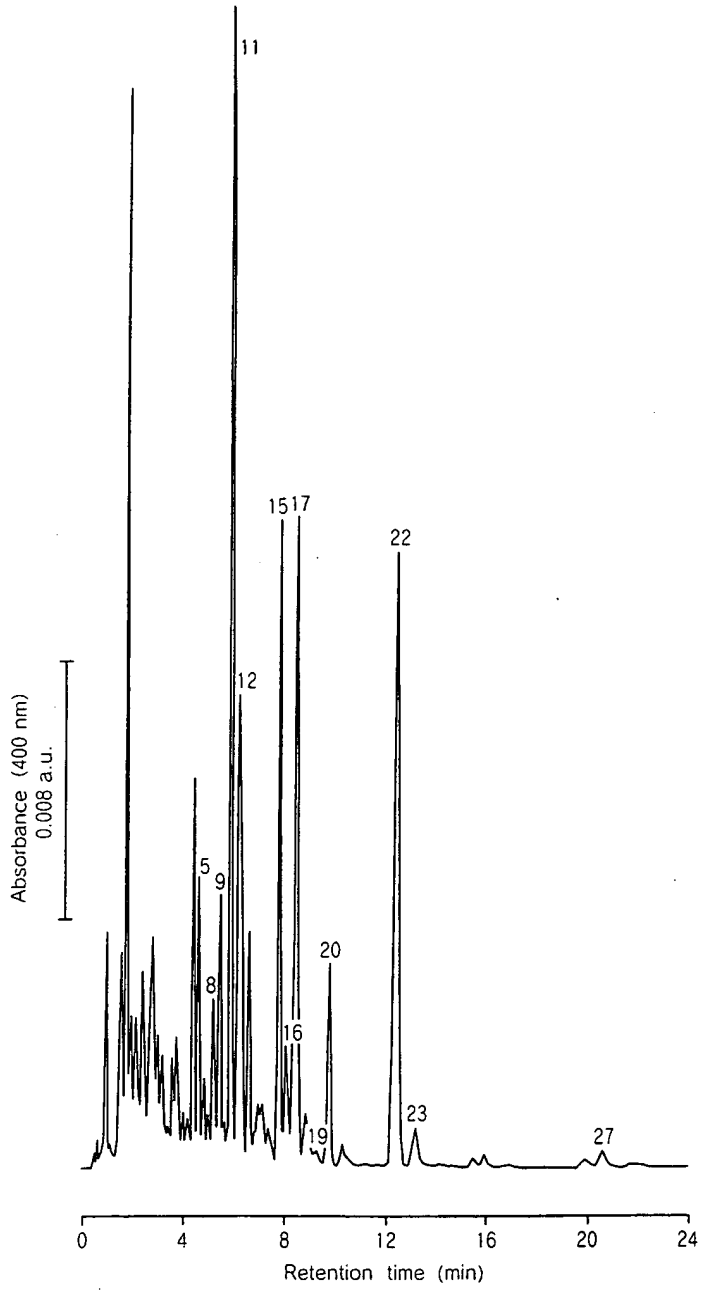

Fig. 9. EFA profile of platelet L-α-phosphatidylethanolamine obtained from a patient with diabetes mellitus after EPA-E administration. Each peak number corresponds to that in Fig. 4.

that the differences in the FA compositions of platelet phospholipids obtained from the patients before and after EPA-E administration were limited mainly to PE. The EPA/AA ratios were significantly increased in all phospholipid classes following oral EPA-E administration.

Needleman et al. [89] described the mechanism of the inhibitory effect of the oral EPA administration on

Table 7
Effect of EPA-E administration on the fatty acid compositions of platelet phospholipids obtained from NIDDM patients[a]

Fatty acid	Fatty acid composition (mol%)							
	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidyl-L-serine		Phosphatidylinositol	
	Before EPA-E	After EPA-E	Before EPA-E	After EPA-E	Before EPA-E	After EPA-E	Before EPA-E	After EPA-E
$C_{14:0}$	0.90 ± 0.30	1.95 ± 3.14	3.25 ± 0.85	3.17 ± 1.02	1.88 ± 1.19	2.33 ± 1.67	2.90 ± 0.96	3.48 ± 1.58
$C_{16:0}$	43.54 ± 3.57	44.70 ± 4.63	14.36 ± 1.93	13.10 ± 1.64**	6.98 ± 3.36	8.06 ± 3.11	10.27 ± 2.27	12.53 ± 3.89
$C_{18:0}$	16.11 ± 2.04	16.16 ± 0.99	32.51 ± 3.73	29.12 ± 4.09*	53.83 ± 6.42	53.03 ± 7.06	54.27 ± 8.16	53.20 ± 8.01
$C_{18:1,cis}$	22.70 ± 3.32	21.45 ± 4.46	11.29 ± 1.13	9.94 ± 1.25*	20.95 ± 5.08	19.11 ± 5.40	4.35 ± 0.70	4.59 ± 1.26
$C_{18:2,cis}$	7.64 ± 1.50	7.89 ± 1.25	8.05 ± 0.48	10.60 ± 2.28**	2.19 ± 0.70	2.39 ± 0.70	4.71 ± 1.14	4.46 ± 1.86
$C_{20:0}$	1.11 ± 0.26	1.19 ± 0.23	1.17 ± 024	0.80 ± 0 14**	2.11 ± 0.20	1.90 ± 0.23	0.63 ± 0.17	0.58 ± 0.14
$C_{20:1}$	1.05 ± 0.30	1.08 ± 020	0.87 ± 0.18	0.72 ± 0.22	0.48 ± 025	0.54 ± 0.21	0.30 ± 0.11	0.36 ± 0.08
$C_{20:2}$	0.35 ± 0.08	0.32 ± 0.12	0.48 ± 0.09	0.62 ± 0.11				
$C_{20:4}$	5.30 ± 0.73	3.71 ± 0.60***	23.47 ± 2.51	24.83 ± 4.72	10.70 ± 2.39	11.79 ± 2.07	22.04 ± 7.83	20.12 ± 4.97
$C_{20:5}$	0.66 ± 0.22	0.94 ± 0.19*	1.16 ± 0.52	3.10 ± 1.33***	0.13 ± 0.07	0.22 ± 0.07*	0.26 ± 0.14	0.47 ± 0.20*
$C_{22:4}$			0.96 ± 0.20	1.24 ± 0.35				
$C_{22:6}$	0.64 ± 0.23	0.61 ± 0.14	2.43 ± 0.72	2.76 ± 0.70	0.75 ± 0.32	0.63 ± 0.38	0.27 ± 0.18	0.21 ± 0.07
EPA/AA	0.125 ± 0.054	0.253 ± 0.063**	0.049 ± 0.021	0.125 ± 0.041***	0.012 ± 0.012	0.019 ± 0.007*	0.012 ± 0.005	0.023 ± 0.008**

[a] Data are expressed as the mean ± S.D. ($n = 10$). The significant difference between the two groups are assessed.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

platelet aggregation. They indicated that oral EPA-E administration was effective at the level of cyclooxygenase to reduce TXA_2 production from AA and, therefore, to decrease the platelet aggregation. Concerning our observations, platelet aggregation rates induced by both ADP and collagen decreased significantly ($P < 0.05$) from 67.6 ± 2.5 and 67.3 ± 3.2 (mean ± S.D.) to 55.7 ± 3.2 and 51.7 ± 4.3 with EPA-E administration, respectively. These results were identical with those in their study and indicated that the EFA compositions incorporated into individual phospholipids of platelet are directly related to the platelet function.

6. Conclusions

The FFAs in serum can be reacted directly with 2-NPH·HCl without hydrolytic or oxidative degradation. This method also allows the direct derivatization of EFAs after saponification of platelet phospholipids, and there are no necessary of cumbersome and rigorous sample work-up steps. The HPLC analysis described here permit the isocratic separation of saturated and mono- and polyunsaturated FAs including *cis–trans* isomers and double-bond positional isomers in samples with good accuracy, precision, selectivity and sensitivity owing to the minimum sample preparation required. In addition, the HPLC method did not produce any artifacts in the procedure involving sample preparation, and improved the column lifetime (more than 500 analyses).

Because of its simplicity and accuracy, the present HPLC method is comparable with routine GC methods for FA analysis and may be successfully used to screen large numbers of biological materials.

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Review

High-performance liquid chromatographic determination of mono-, poly- and hydroxycarboxylic acids in foods and beverages as their 2-nitrophenylhydrazides

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Abstract

The application of direct derivatization in conjunction with high-performance liquid chromatography (HPLC) is described for the determination of both free and total carboxylic acids in foods and beverages. The method is based on the reaction of the carboxylic acids with 2-nitrophenylhydrazine hydrochloride, without complicated isolation steps, which produces their non-volatile hydrazine derivatives. The HPLC of a series of carboxylic acid groups was performed isocratically with short retention times. The analytical results showed good recovery and reproducibility using each internal standard. Due to its excellent selectivity and sensitivity, the present method can serve as a useful tool for routine determinations of mono-, poly- and hydroxycarboxylic acids in foods and beverages.

Keywords: Reviews; Direct derivatization, LC; Food analysis; Milk; Fats; Oils; Fruit juices; Wine; Beer; 2-Nitrophenylhydrazine hydrochloride; Carboxylic acids; Hydroxycarboxylic acids; Fatty acids

Contents

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1. Introduction

The development of liquid chromatographic methods for the routine simultaneous identification and quantification of a variety of carboxylic acids, such as mono-, poly- and hydroxycarboxylic acids, is desirable for use in various fields. Although several chromatographic methods, e.g., ion-exchange and ion-exclusion chromatography, solvophobic chromatography, ion-pair chromatography and reversed-phase chromatography, have been extensively studied for the determination of carboxylic acids, their determination is still an object of research. In the chromatographic methods, carboxylic acids are commonly monitored by refractive index or ultraviolet detection at about 210 nm. This is due to the weak chromophoric properties of the carboxyl group, giving relatively poor sensitivity and selectivity of detection.

To achieve more sensitive and selective detection with high-performance liquid chromatography (HPLC), pre-column derivatization methods have been developed [1–42]. However, most of these methods did not consider quantitative aspects, were not always successful in the series of separations of mono-, poly- and/or hydroxycarboxylic acid derivatives and also need a fairly long analysis time and/or a rigorous sample clean-up procedure. The main problem in the chromatographic methods is that the quantitative isolation of those carboxylic acids is required prior to suitable derivatization. Common isolation procedures involve the use of potassium hydroxide-silicic acid [43], anion-exchange [44] and alumina columns [45]. These procedures may result in loss of sample or in hydrolysis of the endogenous carboxylic acids such as glycerides owing to the relatively long contact with the strong alkali used in the isolation procedures. For determination of esterified carboxylic acids, in addition, extraction is required after saponification to obtain the free carboxylic acids prior to derivatization. Extraction is often tedious and can cause problems with recovery and analytical reliability.

It is therefore desirable to establish a more convenient HPLC method that is rapid and easy to use, involves minimum sample preparation and is suitable for routine analysis.

We have developed the utility of the reagent 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) for the derivatization of mono-, poly- and hydroxycarboxylic acids, and their separation and quantitation by HPLC in various fields [46–62]. The present review demonstrates the direct derivatization of carboxylic acids with 2-NPH·HCl in various foods and beverages without any pre-treatment and/or extraction steps, and the determinations of the acid hydrazides using a reversed-phase HPLC method with simple isocratic elution systems.

2. Derivatization

2.1. Reagent solutions

2-NPH·HCl (Tokyo Kasei Kogyo, Tokyo, Japan) solutions (0.02 *M*) were prepared by dissolving the reagent in water, 0.1 *M* hydrochloric acid–ethanol (1:1, v/v) and 0.3 *M* hydrochloric acid–ethanol (1:1, v/v). A 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC·HCl) (Sigma, St. Louis, MO, USA) solution (0.25 *M*) was prepared by dissolving the reagent in a solution of pyridine (3%, v/v) in ethanol. A potassium hydroxide solution (10%, w/v) in methanol–water (1:1, v/v) and a potassium hydroxide (0.4 *M*)–ethanol (1:1, v/v) solution were prepared. All the reagent solutions were stable for at least 3 months when kept below 5°C, and were also commercially available from Yamamura Chemical Laboratories (Kyoto, Japan). All other chemicals were of analytical-reagent grade, unless stated otherwise.

2.2. Derivatization procedure

Carboxylic acids were dissolved in water, aqueous ethanol or ethanol in various concentrations. To 100 μl of each sample solution, 200 μl of ethanol (if necessary internal standards were added), 200 μl of 2-NPH·HCl in water solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 60°C for 20 min. After the addition of 100 μl of 15% (w/v) potassium hydroxide solution, the mixture was further heated at 60°C for 15 min and then cooled. An aliquot (1–20 μl) of the resulting hydrazide mixture was injected directly into the chromatograph.

2.3. Derivatization conditions

Aqueous, aqueous ethanolic and ethanolic solutions of carboxylic acids react sensitively with 2-NPH·HCl using 1-EDC·HCl as a coupling agent to give non-volatile acid hydrazides [46]. In order to ensure the maximum derivatization of the carboxylic acids, the reaction conditions were investigated with *n*-valeric and myristic acids. Temperature is a very important factor in optimizing the derivatization rate [46]. Investigation of the effect of the temperature on the formation of the acids hydrazides showed that the derivatization rate gradually increased with increasing temperature, but the produced derivatives slightly decreased with reproducible quantitative yields. The peak heights for all carboxylic acids become constant at 3 min and thereafter at 80°C, which suggested that the derivatization was maximal in this period. Using an optimum reaction time of 5 min, the carboxylic acids were converted into their hydrazides without any deterioration [55,56,59,60].

Fig. 1 shows the relationship between the peak heights of the acid hydrazides and the concentration of 2-NPH·HCl. The peak height increased with increase in concentration of 2-NPH·HCl. A 0.02 *M* solution was preferred for the HPLC analyses because a more concentrated solution of the reagent gave impurity peaks on the chromatogram [46].

The effect of the 1-EDC·HCl concentration on the peak height is shown in Fig. 2. Relatively higher peak heights were obtained in the concentration range 0.2–0.3 *M* without affecting the impurity

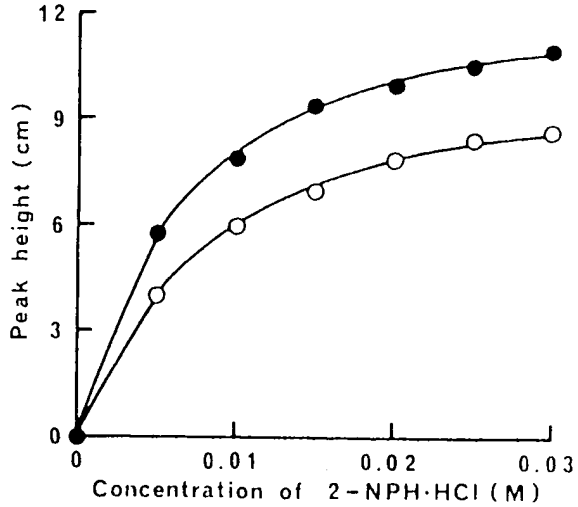

Fig. 1. Effect of concentration of 2-NPH·HCl on colour formation. A 0.8 μmol amount of each acid was treated by the derivatization procedure using various concentrations of 2-NPH·HCl. An aliquot of 2 μl of the reaction mixture was injected into the chromatograph and was detected at 400 nm with $1 \cdot 10^{-2}$ units absorbance range. ●, *n*-Valeric acid; ○, myristic acid.

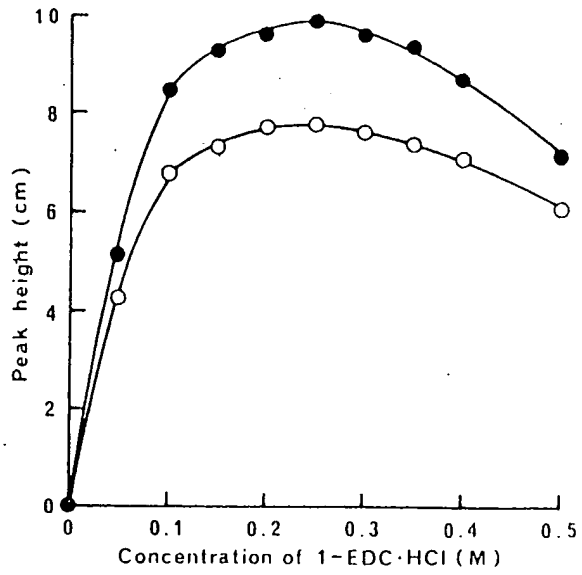

Fig. 2. Effect of concentration of 1-EDC·HCl on colour formation. A 0.8 μmol amount of each acid was treated by the derivatization procedure using various concentrations of 1-EDC·HCl. An aliquot 2 μl of the reaction mixture was injected on into the chromatograph and was detected at 400 nm with $1 \cdot 10^{-2}$ units absorbance range. , *n*-Valeric acid; ○, myristic acid.

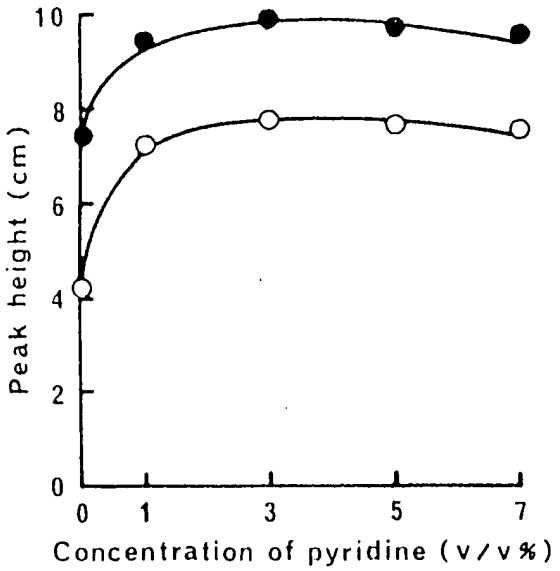

Fig. 3. Effect of concentration of pyridine on colour formation. A 0.8 μmol amount of each acid was treated by the derivatization procedure using various concentrations of pyridine. An aliquot of 2 μl of the reaction mixture was injected into the chromatograph and was detected at 400 nm with $1 \cdot 10^{-2}$ units absorbance range. ●, *n*-Valeric acid; ○, myristic acid.

peaks and 0.25 *M* was selected in subsequent studies [46].

The influence of the concentration of pyridine on the peak heights of the *n*-valeric and myristic derivatives is shown in Fig. 3. The peak heights were almost constant over the range of pyridine concentrations investigated. These experiments indicated that the optimum concentration of pyridine was 3% (v/v) [46].

A concentration of 10% (w/v) potassium hydroxide was needed in order to eliminate the interference in the chromatogram due to the excess of the reagents and the reaction by-products that might be formed during the coupling reaction [46]. In this derivatization process, polycarboxylic acids converted to their monohydrazine derivatives, i.e., acidic acid compounds due to the residual carboxyl group [47,53,54,60].

The absorption curves of acetic acid hydrazide in solutions of various pH are shown in Fig. 4. The hydrazide ionizes at high pH (>12) to give an intense violet colour. At low pH (<8.5), however, the absorption maximum of the hydrazide shifts considerably towards the blue region. On the other

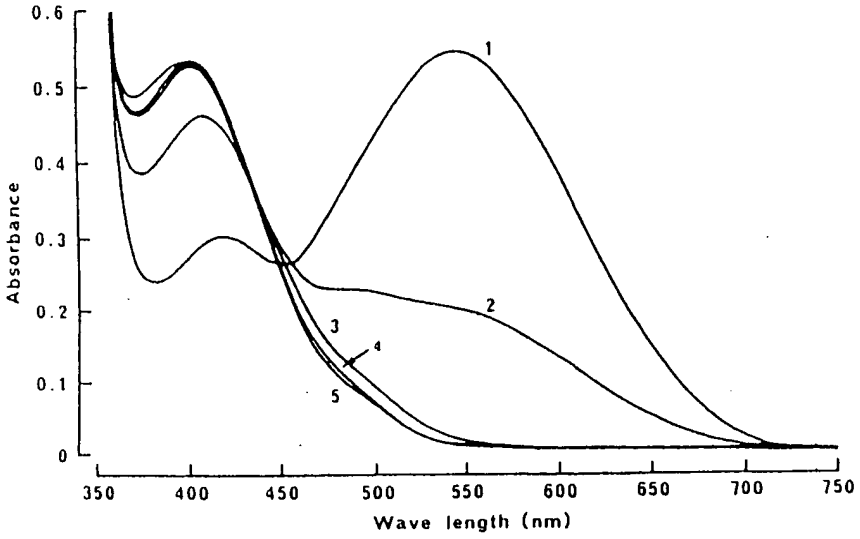

Fig. 4. Absorption spectra of solutions of acetic acid 2-nitrophenylhydrazine of various pH. A 0.5 μmol amount of acetic acid was treated by the derivatization procedure. The reaction mixture was adjusted with 3 *M* HCl to the desired pH and was measured from 350 to 750 nm. pH: 1=13.85; 2=10.5; 3=8.5; 4=6.5; 5=4.5.

hand, with the reversed-phase HPLC column the pH of the eluent was restricted to the range 2–8, and the elution system of mixtures of methanol, acetonitrile and water was chosen to maintain the pH at 4–7. All of the carboxylic acid hydrazides gave absorption maxima at 400 nm in the acidic medium, and were detectable photometrically by monitoring at this wavelength. These derivatives also showed strong absorption in the UV region, with maximal absorption at around 230 nm, and were monitored with a UV detector. An excess of the reagents and the reaction by-products did not interfere with the HPLC analyses in the visible range, because they did not absorb visible radiation at 400 nm and were eluted before any of the carboxylic acid hydrazides. The advantage of using visible detection is that the chromatograms are simpler and more selective, in spite of approximately four-fold lower sensitively than when UV detection is used [46,51].

This derivatization procedure with slightly modification is used to the direct derivatization of various carboxylic acids, such as mono-, poly- and hydroxycarboxylic acids, in foods and beverages without conventional isolation steps [48,55,58–60]. The major problems arising in the direct derivatization of carboxylic acids in foods and beverages are the presence of protein-bound acids and the pH of the reaction mixture. Deproteinization was easily accomplished by the ethanol in the reaction mixture, and the protein-bound acids could be converted into their hydrazides, because the amount of ethanol (ca. 71.4%, v/v) was sufficient to denature and precipitate the protein [50,51,55]. Another problem is that the pH of the reaction mixture was slightly increased by basic substances occurring the foods and beverages, resulting in a decrease of the yields of the carboxylic acid hydrazides. Therefore, 2-NPH·HCl was dissolved in 100 m*M* hydrochloric acid–ethanol (1:1, v/v) instead of water to obtain maximum yields of the hydrazides. The carboxylic acid profile was unaffected by the amount of hydrochloric acid [50,51,55]. These experiments established that the carboxylic acids in foods and beverages could be directly converted into their hydrazides in high yields. The carboxylic acids derivatized with 2-NPH·HCl were classified for each purpose into the two groups, short-chain fatty acids (SCFAs) and long-chain fatty acids (LCFAs) [50,51,55].

2.4. Assay of free fatty acids in milk and milk products

For samples 100 μl of milk, about 20 mg of butter and cheese and about 50 mg of condensed milk, ice cream and yogurt were measured exactly. To the milk product samples 100 μl of water was added. To each sample solution, 200 μl of ethanol containing 20 nmol of 2-ethylbutyric acid and 20 nmol of margaric acid as internal standards, 200 μl of 2-NPH·HCl in 0.1 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. The resulting hydrazide mixture was neutralized by adding 4 ml of 1/30 *M* phosphate buffer (pH 6.4)–0.5 *M* hydrochloric acid (7:1, v/v) and the long-chain free fatty acid (LCFFA) hydrazides were extracted with 5 ml of *n*-hexane. About a 3 ml portion of the residual aqueous layer was taken and the short-chain free fatty acid (SCFFA) hydrazides were extracted twice with 4 ml of diethyl ether. The *n*-hexane layer and the combined ether layer were evaporated with a stream of nitrogen at room temperature. Each residue was dissolved in 200 μl of methanol and an aliquot of 10–20 μl was injected into the chromatograph.

2.5. Assay of total fatty acids in milk and milk products

For samples, 10 μl of milk, about 1 mg of butter and cheese, about 2 mg of condensed milk and ice cream and about 10 mg of yogurt were measured exactly. Each sample was dissolved in 200 μl of ethanol containing 400 nmol of 2-ethylbutyric acid and 200 nmol of margaric acid as internal standards and was saponified with 100 μl of 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) solution at 80°C for 20 min. To the saponified sample, 200 μl of 2-NPH·HCl in 0.3 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. The resulting hydrazide mixture was treated in the same way as in the de-

termination of free fatty acids (FFAs) in milk and milk products.

The *n*-hexane layer and combined ether layer were evaporated with a stream of nitrogen at room temperature. Each residue was then dissolved in 200 μl of methanol and an aliquot of 2–10 μl was injected into the chromatograph.

2.6. Assay of long-chain total fatty acids in fats and oils

About 1 mg of fat and oil samples were measured exactly. Each sample was dissolved in 200 μl of ethanol containing 400 nmol margaric acid as internal standard and saponified with 100 μl of 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) at 80°C for 20 min. To the saponified sample, 200 μl of 2-NPH·HCl in 0.3 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution was added. The mixture was heated at 80°C for 5 min. After addition of 200 μl of 10% (w/v) potassium hydroxide, the mixture was further heated at 80°C for 5 min and then cooled. The hydrazide mixture (5–10 μl) was injected into the chromatograph.

2.7. Assay of mono-, poly- and hydroxycarboxylic acids in beverages

For samples, 50 μl of wines, 25 μl of fruit juices, 100 μl of beer and 50 μl of Japanese "sake" were exactly measured and each sample, with the exception of beer, was diluted with water to 100 μl. To each sample solution, 200 μl of ethanol containing 400 nmol of 3-methylglutaric acid as internal standard, 200 μl of 2-NPH·HCl in 0.1 *M* hydrochloric acid–ethanol (1:1, v/v) solution and 200 μl of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 μl of 10% (w/v) potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. The hydrazide mixture (5–10 μl) was injected into the chromatograph.

3. Chromatographic analysis

3.1. Instrumentation

Chromatographic analyses were carried out using a Shimadzu LC-6A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an on-line degasser ERC-3310 (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV–visible detector. The detector signals were recorded on a Rikadenki multi-pen recorder (Tokyo, Japan). The column temperature was kept constant at 30–50°C using a Shimadzu GTO-6A column oven. All columns were packed at Yamamura Chemical Laboratories.

3.2. HPLC conditions

The separation of 10 SCFA hydrazides was achieved on a YMC-FA (C_8) main column (particle size 5 μm, 250×6 mm I.D.) with a BBC-4-C_8 guard column (particle size 5 μm, 10×4 mm I.D.). The eluent was maintained at about pH 4–5 by adding 0.1 *M* hydrochloric acid and was filtered through a Nuclepore filter (pore size 2 μm) (Nomura Micro Science, Osaka, Japan).

The separation of 29 LCFA hydrazides was achieved on a J'sphere ODS-M 80 main column (particle size 4 μm, 250×4.6 mm I.D.) with a guard cartridge (J'sphere ODS-M 80). The eluent was maintained at about pH 4–5 by adding 0.1 *M* hydrochloric acid and was filtered through a Fluoropore filter (pore size 0.45 μm) (Sumitomo Electric, Osaka, Japan).

The separation of 10 carboxylic acid hydrazides was carried out on a J'sphere ODS-M 80 main column (particle size 4 μm, 250×6 mm I.D.) with a BBC-5-C_8 guard column (particle size 5 μm, 10×5 mm I.D.). The pH was adjusted to the desired value by mixing 0.005 *M* KH_2PO_4–acetonitrile–methanol with 0.005 *M* Na_2PO_4–acetonitrile–methanol and then dissolving counter-ions at a concentration of 0.005 *M*. The counter-ions studied were tetramethylammonium, tetraethylammonium and tetra-*n*-propylammonium (TMA, TEA and TPA, respectively) as their bromides. The eluent was filtered through a Nucleopore filter (pore size 0.2 μm) (Nomura Micro Science).

3.3. HPLC separation

Chromatographic separations of fatty acid derivatives are usually performed on reversed-phase columns with isocratic or gradient elution systems

comprising acetonitrile, methanol, and water in various proportions. The elution volumes of the fatty acid derivatives are affected principally by the number of carbon atoms and the number of unsaturated bonds in the fatty acid chains [48,50,55,56]. Acetonitrile and methanol have different effects on the two parameters. Therefore, the conditions for HPLC separations of the SCFA and LCFA hydrazides were investigated using the YMC-FA (C_8) column and/or the J'sphere ODS-M 80 column with different isocratic eluents consisting of acetonitrile, methanol and water in various proportions.

Fig. 5 shows a typical separation of 10 SCFA hydrazides ($C_{2:0}$–$C_{6:0}$) including iso isomers and

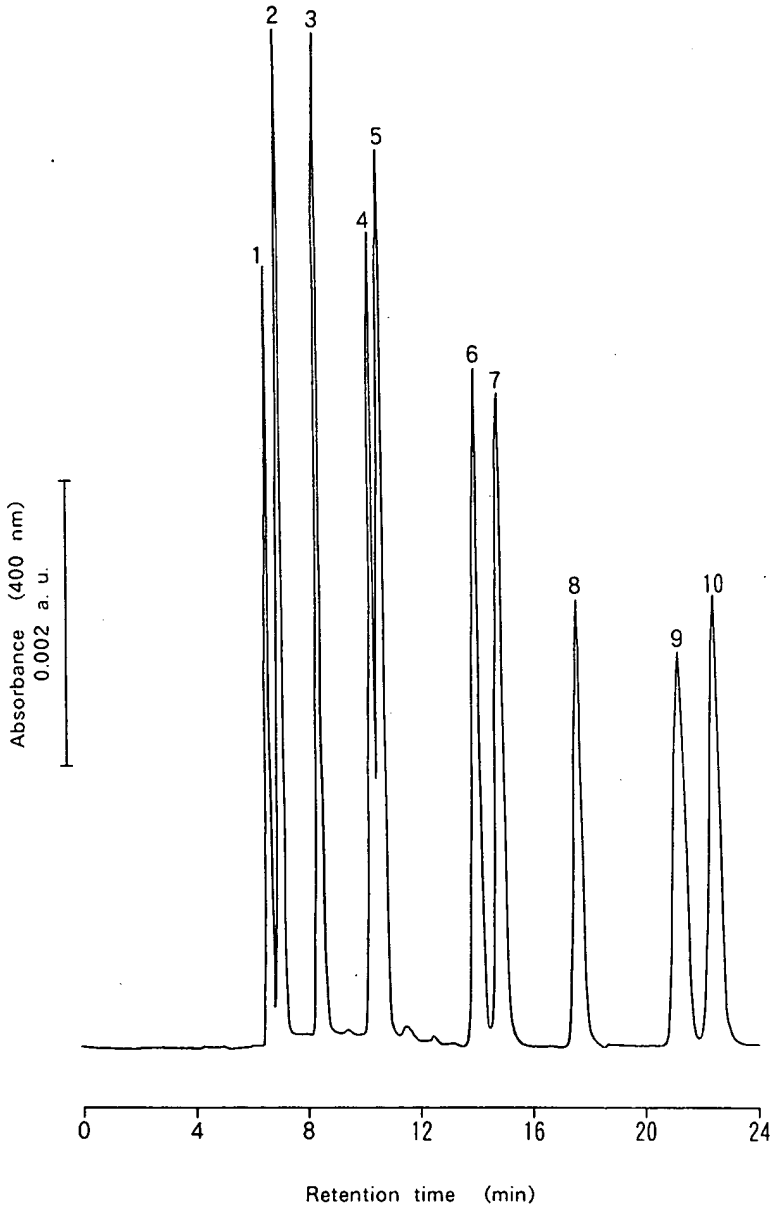

Fig. 5. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 10 fatty acids obtained with visible detection. Peaks: 1=lactic; 2=acetic; 3=propionic; 4=isobutyric; 5=*n*-butyric; 6=isovaleric; 7=*n*-valeric; 8=2-ethylbutyric (internal standard); 9=isocaproic; 10=*n*-caproic acid hydrazide. Each peak corresponds to 150 pmol.

lactic acid hydrazide by HPLC with acetonitrile–methanol–water (30:20:50, v/v/v) as the eluent at a flow-rate of 1.2 ml/min. The column temperature was maintained at 30°C. The iso isomers were eluted faster than the normal isomers [46,50,55].

Fig. 6 shows a chromatogram of a mixture of saturated and mono- and polyunsaturated LCFA hydrazides ($C_{8:0}$–$C_{22:6}$), including *cis–trans* isomers, obtained by using acetonitrile–water (86:14, v/v) as the eluent at a flow-rate of 2.0 ml/min. The retention times increased with increasing chain length for the LCFA hydrazides and inversely with the degree of unsaturation for the unsaturated LCFA hydrazides. The *trans* isomers were eluted after the corresponding *cis* isomers [55,56,59,61]. The chromatographic behaviour of the hydrazine derivatives agree with those of other FA derivatives, and lead to the occurrence of several pairs of fatty acids which are difficult to separate.

However, resolution of double-bond positional isomers, such as γ-linolenic (*n*-6) and α-linolenic acid (*n*-3) hydrazides and *n*-9, *n*-12, and *n*-15 eicosenoic acid hydrazides, is not always achieved. Our previous works [55,56,59,61] showed that acetonitrile has a significant effect on the two factors affecting the elution volumes of fatty acid hydrazides. By increasing the proportion of acetonitrile in the eluent, γ-linolenic (*n*-6) and myristic acid hydrazides were resolved, as were docosahexaenoic (*n*-3) and palmitoleic acid (*n*-7) hydrazides. This change, however, also resulted in decreased resolutions of myristoleic (*n*-5) and octadecatetraenoic acid (*n*-3) hydrazides and of palmitic and docosatetraenoic acid (*n*-6) hydrazides and of oleic (*n*-9) and elaidic acid (*n*-9) hydrazides. Column temperature also has a significant effect on resolution. Increasing the column temperature from 30°C to 50°C leads to inversion in selectivity. Therefore, increasing column temperature caused greater resolutions of the latter three critical pairs but loss of resolutions of the former two critical pairs. For this study, column temperature was set at 50°C to shorten analysis time and yet achieve good resolution. However, resolution of eicosatrienoic (*n*-3) and dihomo-γ-linolenic acid (*n*-6) hydrazides could not be achieved. Separation of 29 LCFA hydrazides is performed within only 22 min by elution in the isocratic mode, which is a distinct advantage over gradient elution techniques [59,61]. The same separation has hitherto been achieved only with capillary gas chromatography.

Reversed-phase ion-pair chromatography (RP-IPC), in which a hydrophobic stationary phase and an aqueous buffer containing a low concentration of counter-ion are used, facilitates the separation of both ionized and non-ionized compounds under the same chromatographic conditions. Our previous works [47,53,54,60] showed that mono-, poly- and hydroxycarboxylic acids were selectively separated as their 2-nitrophenylhydrazides on RP-IPC by the influence of pH, the polarity of mobile phase, and the size of counter-ion.

A pH of 7 was chosen to convert the acidic acid hydrazides into their ionized forms. A small-size ion-pair reagent should be selected to not dominate the chromatographic behaviour of the ion-pair reagent. The eluents containing quaternary alkyl ammonium as their bromide compounds ranging from TMA to TPA were prepared by mixing known volumes of acetonitrile, methanol and aqueous phosphate buffers. When TPA was used as the counter-ion, large retention volumes were observed with a significant loss of resolution. TMA and TEA gave similar resolution, but TEA was selected as the optimum counter-ion since it yielded a higher capacity factor with no loss in resolution. The column temperature has a significant effect on the separation of various pairs of carboxylic acids and an increase from 30°C to 50°C leads to an inversion in the selectivity [60]. By increasing the temperature separation of L-pyroglutamic and lactic and acetic acid hydrazides could be achieved, but this change also resulted in a decreased separation of tartaric and malic and succinic acid hydrazides. Vice versa, decreasing the temperature caused greater separation of these acidic acid hydrazides, but resulted in a loss of separation between lactic and acetic acids hydrazides.

Fig. 7 shows a typical chromatogram of 10 mono-, poly- and hydroxycarboxylic acid hydrazides by RP-IPC analysis with phosphate buffer–acetonitrile–methanol (80:10:10, v/v) containing 0.005 *M* TEA as the isocratic eluent at a flow-rate of 2.0 ml/min. The column temperature was maintained at 35°C. In the chromatogram, two peaks appeared for both citric and malic acid hydrazides: these are attributed to stereochemical isomers of the derivatives [60].

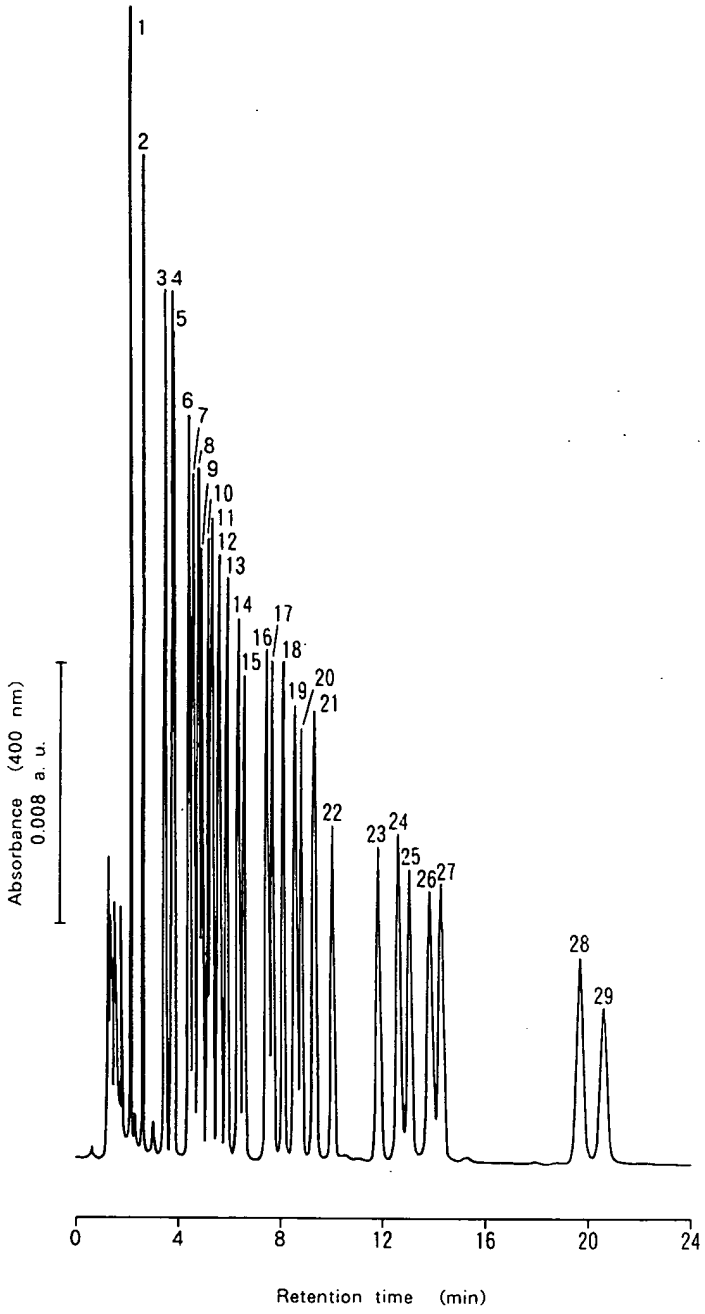

Fig. 6. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 29 fatty acids obtained with visible detection. Peaks: 1=caprylic ($C_{8:0}$); 2=capric ($C_{10:0}$); 3=lauric ($C_{12:0}$); 4=myristoleic ($C_{14:1}$, *n*-5); 5=octadecatetraenoic ($C_{18:4}$); 6=eicosapentaenoic ($C_{20:5}$, *n*-3); 7=α-linolenic ($C_{18:3}$, *n*-3); 8=γ-linolenic ($C_{18:3}$, *n*-6); 9=myristic ($C_{14:0}$); 10=docosahexaenoic ($C_{22:6}$, *n*-3); 11=palmitoleic ($C_{16:1}$, *n*-7); 12=arachidonic ($C_{20:4}$, *n*-6); 13=linoleic ($C_{18:2,cis,cis}$, *n*-6); 14=linoelaidic ($C_{18:2,trans,trans}$, *n*-6); 15=eicosatrienoic ($C_{20:3}$, *n*-3) and dihomo-γ-linolenic ($C_{20:3}$, *n*-6); 16=palmitic ($C_{16:0}$); 17=docosatetraenoic ($C_{22:4}$, *n*-6); 18=oleic ($C_{18:1,cis}$, *n*-9); 19=elaidic ($C_{18:1,trans}$, *n*-9); 20=eicosadienoic ($C_{20:2}$, *n*-6); 21=margaric ($C_{17:0}$) (internal standard); 22=docosatrienoic ($C_{22:3}$, *n*-3); 23=stearic ($C_{18:0}$); 24=eicosenoic ($C_{20:1}$, *n*-9); 25=eicosenoic ($C_{20:1}$, *n*-12); 26=docosadienoic ($C_{22:2}$, *n*-6); 27=eicosenoic ($C_{20:1}$, *n*-15); 28=arachidic ($C_{20:0}$); 29=erucic ($C_{22:1}$, *n*-9) acid hydrazide. Each peak corresponds to 150 pmol.

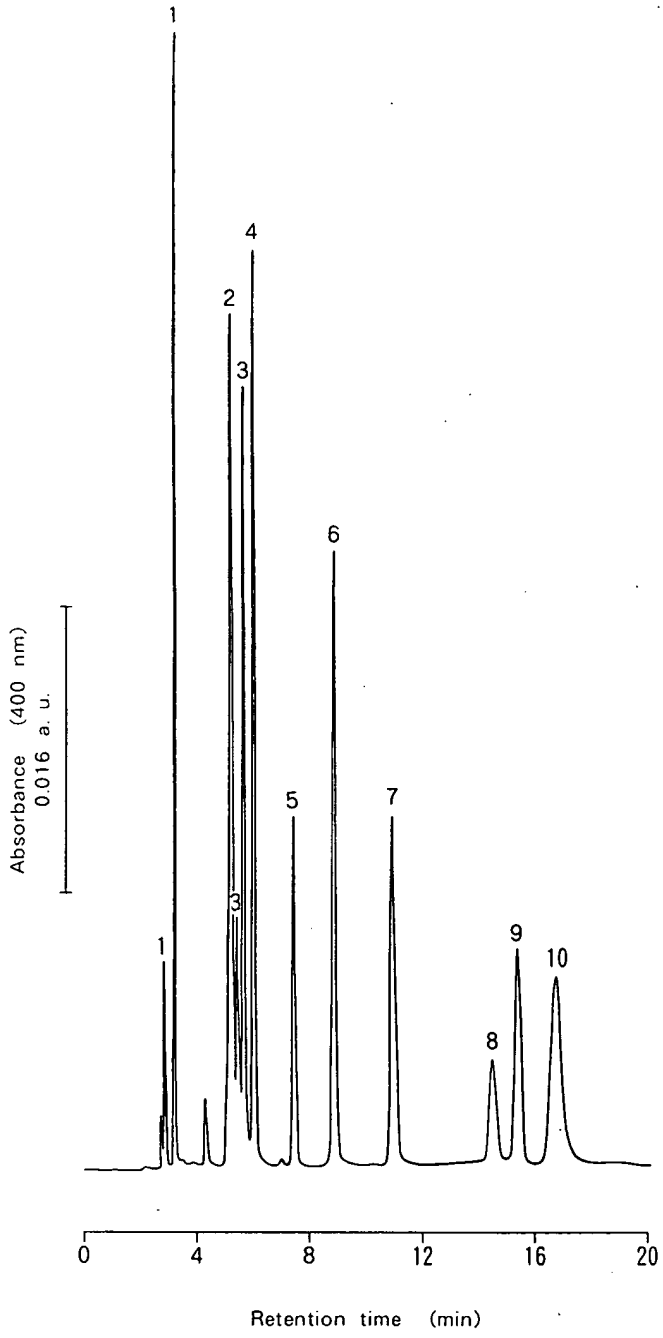

Fig. 7. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of mono-, poly- and hydroxycarboxylic acids obtained with visible detection. Peaks: 1=citric; 2=tartaric; 3=malic; 4=succinic; 5=fumalic; 6=3-methylglutaric (internal standard); 7=glycolic; 8=L-pyroglutamic; 9=lactic; 10=acetic acid hydrazide. Each peak corresponds to 1 nmol.

4. Quantitative analysis

4.1. Calculation

Calibration curves were constructed by derivatizing increasing amounts of carboxylic acids in the presence of internal standard and analysing as described above. The calibration test was replicated five times. From the chromatograms obtained, the relationships between the peak-height ratios of the acid hydrazides to that of internal standard and the concentrations of the acids were calculated by the least-squares method. Previous works [48,55,59,60] demonstrated that the calibration curves of individual carboxylic acids were linear over a wide concentration range with good correlation coefficients (0.999–1.000). The limits of detection, based on a signal-to-noise ratio of 2, were 500 fmol–4 pmol per injection.

4.2. Recovery and precision

The recovery and reproducibility for the assay of free fatty acids (FFAs) and total fatty acids (TFAs) in milk and milk products were investigated six times in butter by adding known mixtures of the SCFAs and LCFAs. In this experiment, the recoveries of FFAs were in the range 95.2–104.3% [relative standard deviations (RSDs)=1.1–4.2%], when the following amounts of fatty acids were added to 20 mg of the butter: $C_{4:0}$, $C_{6:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$=20 nmol; others=5 nmol. The recoveries of TFAs were in the range 96.4–103.3% (RSD=0.5–3.9%), when the following amounts of fatty acids were added to 1 mg of the butter: $C_{4:0}$, $C_{6:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$=200 nmol; others=50 nmol. The inter-assay precision was evaluated by assaying nine times the same butter sample for FFAs and TFAs. The RSDs ranged from 0.4 to 4.5% and from 0.6 to 3.7% for the FFAs and TFAs, respectively.

In order to examine the recovery and reproducibility for the assay of LCTFAs in fats and oils known amounts (50 and 200 nmol) of the fatty acid mixtures were added to triglycerides (TGs) from pig liver (1 mg). Each aliquot was analysed by nine separate measurements. The recoveries of fatty acids were in the range 97.3–102.8% (RSD=0.5–2.4%) and 97.9–103.1% (RSD=0.6–2.9%), respectively, for 50 and 200 nmol of added fatty acids. The intra-assay precision was evaluated by assaying the same TGs six times. The inter-assay precision was determined by analyzing spiked TGs on different days over 1 week (n=6). The intra- and inter-assay precisions ranged from 0.4 to 2.0% and from 0.5 to 2.6%, respectively.

To determine the precision for the assay of mono-, poly- and hydroxycarboxylic acids in beverages known amounts (50 and 200 nmol) of the carboxylic acid mixture was added to pooled red wine (50 μl). Each aliquot was analysed nine times. The recoveries of the carboxylic acids were 97.5–103.9% (RSD=0.9–3.1%) and 98.7–102.8% (RSD=0.8–3.7%) for 50 and 200 nmol, respectively. The intra-assay precision was evaluated by assaying the same red wine six times. The inter-assay precision was determined by analyzing spiked red wine on different days over 1 week (n=6). The intra- and inter-assay precisions ranged from 0.7 to 2.8% and from 0.5 to 3.7%, respectively. These results indicate that the method has a satisfactory precision and reproducibility for the determination of various carboxylic acids in foods and beverages [55,59,60].

5. Application

5.1. Determination of free fatty acids and total fatty acids in milk and milk products

FFAs in milk and milk products contribute to their desirable flavour, but when present in excessive amounts can impart a rancid flavour. Elevated FFA levels are generally caused by the natural milk lipase and/or by the heat-stable bacterial lipase [63]. The determination of TFAs, i.e., the sum of FFAs and esterified fatty acids (EFAs) in milk and milk products, is necessary to investigate whether they are contaminated with other fats and oils.

The FFA and TFA profiles of SCFAs in butter are shown in Figs. 8 and 9, respectively. In the HPLC of SCFFAs and SCTFAs some unknown peaks were found on the chromatograms, but the hydrazides

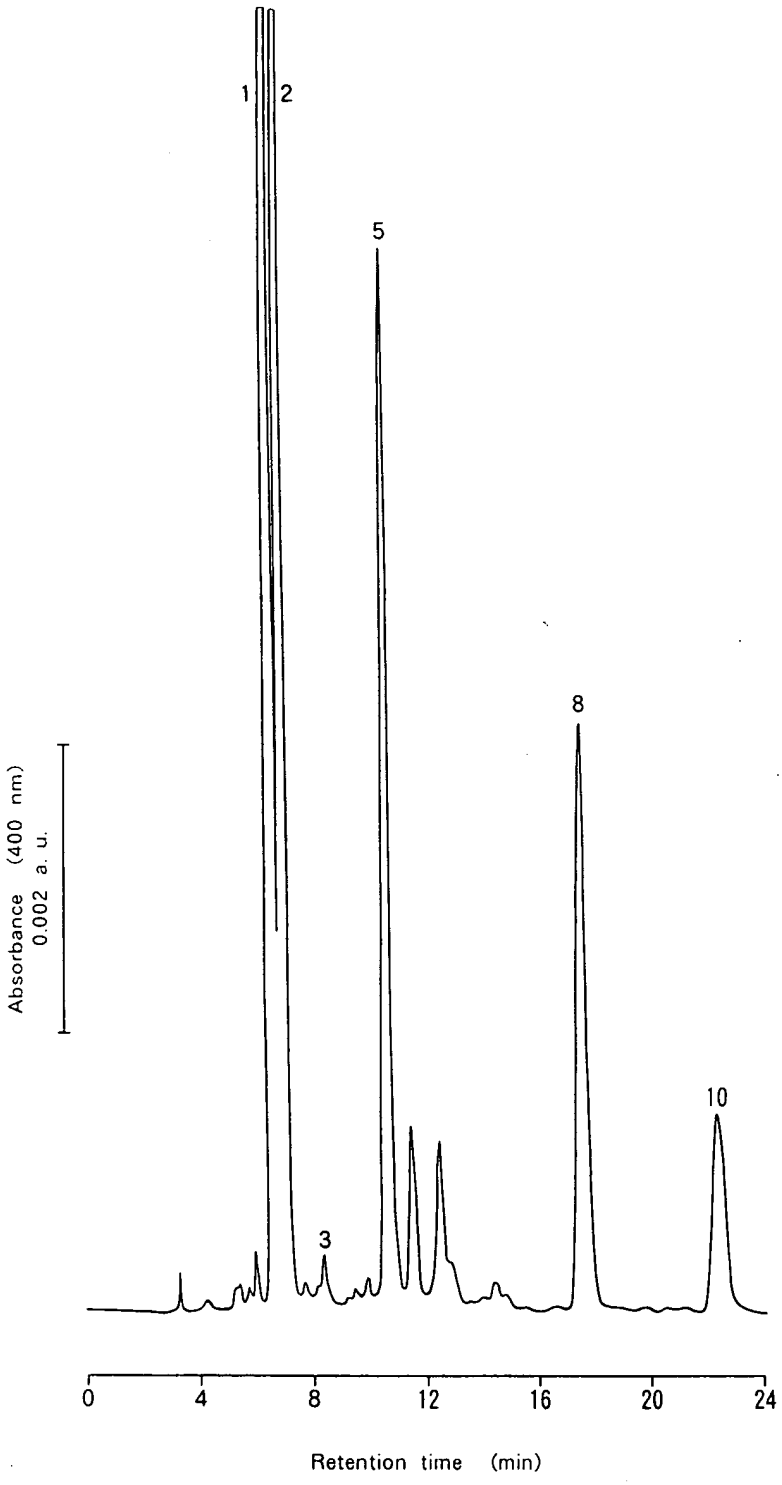

Fig. 8. Short-chain free fatty acid profile of butter. Each peak number corresponds to that in Fig. 5.

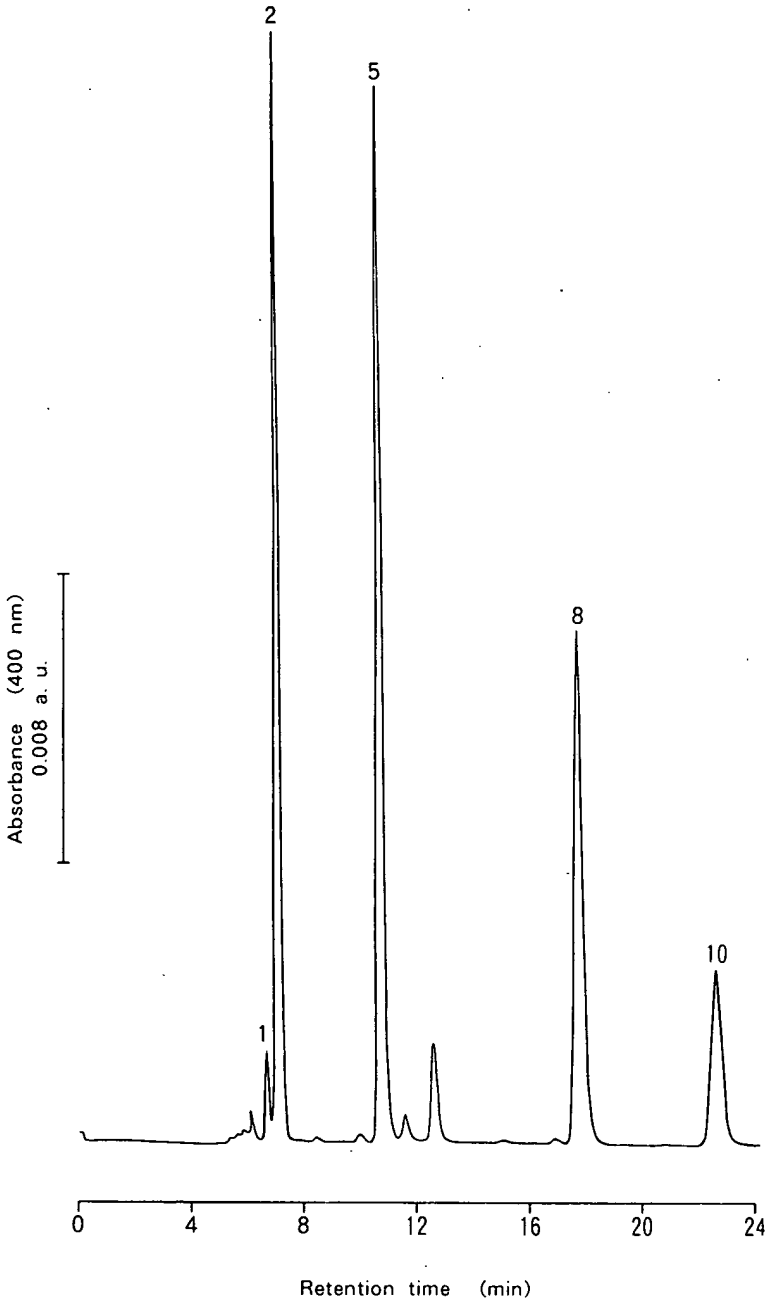

Fig. 9. Short-chain total fatty acid profile of butter. Each peak number corresponds to that in Fig. 5.

were separated from the unknown peaks, with the exception of acetic acid hydrazide. All the fatty acids in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards, because the fatty acids derived from milk and milk products varied from C_4 to C_{20} [43–45,64–67].

Tables 1 and 2 give the amounts of SCFFA and

Table 1
Amounts of SCFFAs in milk and milk products

SCFFA	Amount of SCFFA (nmol/g)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
Lactic	459.10[b]	733.75	5.39[c]	80.03[c]	649.50	64.26[c]
n-Butyric	162.89[b]	128.17	873.95	532.21	51.39	112.49
n-Caproic	39.20[b]	41.60	328.00	168.00	18.80	40.00

[a] Mean results ($n=3$).
[b] nmol/ml.
[c] μmol/g.

Table 2
Amounts of SCTFAs in milk and milk products

SCTFA	Amount of SCTFA (nmol/g)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
Lactic	15.29[b]	28.43	38.55	194.13	85.67	88.63
n-Butyric	15.55[b]	31.78	484.99	235.58	20.45	9.20
n-Caproic	5.29[b]	10.60	165.09	81.04	24.58	2.95

[a] Mean results ($n=3$).
[b] μmol/ml.

Table 3
LCFFA compositions of milk and milk products

LCFFA	LCFFA compositions (mol %)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
$C_{8:0}$	3.69	3.76	1.48	1.78	1.57	5.05
$C_{10:0}$	5.77	5.59	3.58	3.90	4.75	5.71
$C_{12:0}$	5.69	5.56	5.78	6.99	5.07	6.05
$C_{14:0}$	12.16	12.07	14.27	11.81	10.68	14.18
$C_{14:1}$	1.34	1.39	1.36	1.00	0.79	1.12
$C_{16:0}$	28.32	26.64	31.88	26.68	28.57	31.43
$C_{16:1}$	1.93	2.15	2.08	1.76	1.52	1.44
$C_{18:0}$	9.34	8.83	9.91	13.99	22.64	9.34
$C_{18:1,\omega 9}$	25.15	25.97	22.71	16.79	19.69	20.54
$C_{18:2,\omega 6,\omega 6}$ (*n*-6)	4.87	5.91	5.03	12.16	3.34	3.70
$C_{18:3}$ (*n*-3)	0.53	0.65	0.58	1.45	1.38	0.44
$C_{20:2}$ (*n*-6)	0.57	0.53	0.48			
$C_{20:3}$ (*n*-6)	0.24	0.21	0.20	0.20		
$C_{20:4}$ (*n*-6)	0.40	0.74	0.66	1.49		1.00
Total (μmol/g)	1.013[b]	1.680	9.554	6.899	1.281	0.560

[a] Mean results ($n=3$).
[b] μmol/ml.

SCTFA in milk and milk products. The SCFFAs, butyric acid ($C_{4:0}$) and caproic acid ($C_{6:0}$) are largely responsible for the rancid flavour, and the total $C_{4:0}$/$C_{6:0}$ ratio is very significant for determining the criterion of contamination. Tables 1 and 2 indicate that there is a large difference between the free and total lactic acid estimates. One possible explanation for the difference is the decomposition of lactose that

Table 4
LCTFA compositions of milk and milk products

LCTFA	LCTFA compositions (mol %)[a]					
	Milk	Condensed milk	Butter	Cheese	Ice cream	Yogurt
$C_{8:0}$	3.43	3.20	3.76	3.19	4.18	3.31
$C_{10:0}$	6.85	5.65	7.04	6.01	7.45	5.68
$C_{12:0}$	5.81	5.31	6.51	6.29	6.54	5.22
$C_{14:0}$	16.78	16.31	18.04	16.78	16.06	15.70
$C_{14:1}$	1.29	1.49	1.59	1.22	1.11	1.27
$C_{16:0}$	30.44	29.98	31.62	29.81	28.18	30.73
$C_{16:1}$	1.50	1.82	1.57	1.45	1.37	1.58
$C_{18:0}$	11.16	10.42	9.45	11.90	13.76	11.80
$C_{18:1,cis}$	17.23	20.15	15.33	18.12	16.34	18.65
$C_{18:2,cis,cis}$ (*n*-6)	3.90	3.80	3.38	3.35	2.64	4.15
$C_{18:3}$ (*n*-3)	0.39	0.34	0.34	0.69	1.11	0.29
$C_{20:2}$ (*n*-6)	0.14	0.19	0.19	0.10	0.12	0.30
$C_{20:3}$ (*n*-6)	0.24	0.31	0.24	0.24	0.23	0.32
$C_{20:4}$ (*n*-6)	0.93	1.03	0.94	0.85	0.91	1.00
Total (mmol/g)	0.140[b]	0.334	2.300	0.938	0.454	0.088

[a] Mean results ($n=3$).
[b] mmol/ml.

Table 5
Fatty acid compositions of fats and oils[a]

Fatty acid	Fatty acid composition (mol %)								
	Vegetable						Animal		Fish,
	Coconut	Olive	Soybeen	Corn	Safflower	Margarine	Beef tallow	Lard	Sardine
$C_{8:0}$	6.30								
$C_{10:0}$	5.28						0.08	0.18	
$C_{12:0}$	50.06					0.74	0.13	0.16	1.03
$C_{14:0}$	20.51					2.83	2.82	2.89	11.57
$C_{14:1}$ (*n*-5)							0.35	0.27	3.92
$C_{16:0}$	7.93	11.85	8.91	12.52	10.20	35.39	31.77	28.96	19.17
$C_{16:1}$ (*n*-7)	0.63						1.71	3.35	10.21
$C_{18:0}$	2.68	4.28	3.72	1.80	2.25	7.29	27.79	13.88	2.70
$C_{18:1,cis}$ (*n*-9)	5.31	75.58	23.73	25.30	11.69	39.61	29.92	37.14	11.64
$C_{18:1,trans}$ (*n*-9)							0.86	0.76	
$C_{18:2,cis,cis}$ (*n*-6)	1.93	7.01	54.24	58.72	74.12	13.42	3.04	10.85	3.48
$C_{18:3}$ (*n*-3)	0.65	7.71	1.66	1.74	0.08	0.54	0.30	0.90	
$C_{18:3}$ (*n*-6)		1.69							
$C_{18:4}$ (*n*-3)									0.12
$C_{20:1}$ (*n*-9)					0.64		0.57	2.34	
$C_{20:2}$ (*n*-6)						0.42	0.42	0.30	
$C_{20:3}$ (*n*-3 and 6)						0.57	0.45		
$C_{20:4}$ (*n*-6)									0.82
$C_{20:5}$ (*n*-3)									16.17
$C_{22:1}$ (*n*-9)									4.11
$C_{22:2}$ (*n*-6)									0.28
$C_{22:6}$ (*n*-3)									11.24
Total (mmol/g)	5.897	2.281	2.462	2.482	2.637	2.006	2.682	2.770	2.495

[a] Data are expressed as the mean ($n=3$).

may be present in the samples by heating with potassium hydroxide. Other differences between the SCFFAs and SCTFAs were accounted for the EFAs. The fatty acid compositions of LCFFAs and LCTFAs in the samples are given in Tables 3 and 4. In all the samples, the chain length of the LCFAs extends from C_8 to C_{20}, and the amounts of LCTFAs were at least 100-times higher than those of LCFFAs. The individual levels of SCFFAs and LCFFAs in the butter sample were consistent with those reported by Wood and Lindsay [67].

5.2. Determination of long-chain total fatty acids in fats and oils

The biological effects of routinely consumed fats and oils are of wide interest because of their impact on human health and nutrition [68,69]. In particular, the ratio of *n*-3 polyunsaturated fatty acids to *n*-6 polyunsaturated fatty acids (*n*-3/*n*-6) seems to be associated with atherosclerosis and breast and colon cancers [70–74].

The method was used to quantitate LCTFAs in some common edible fats and oils (Figs. 10 and 11). The chromatograms, monitored by visible absorbance, showed very clean backgrounds. Thus, LCFAs in samples were easily identified by comparison of retention times of their hydrazides with those of standards. The peak labelled "X" in Fig. 11 is still unknown. This fatty acid may be an isomer of eicosenoic acid (*n*-11), on the basis of the retention behaviour of the other positional isomers (*n*-9, *n*-12, and *n*-15). Table 5 compares the major TFAs of vegetable and fish oils and animal fats. Vegetable oils differ among themselves in the percentage of C_{16} to C_{18} fatty acids, with the exception of coconut oil. In animal fats, fatty acid chain lengths extend from C_{10} to C_{20}. Sardine oil has similar fatty acids as other edible fats and oils and also contains longer chain *n*-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexanoic acid. Table 5 indicates that the principal polyunsaturated fatty acids of sardine oil are in the *n*-3 family.

The data obtained by the present method were in good agreement with those of other reports [11,48,74].

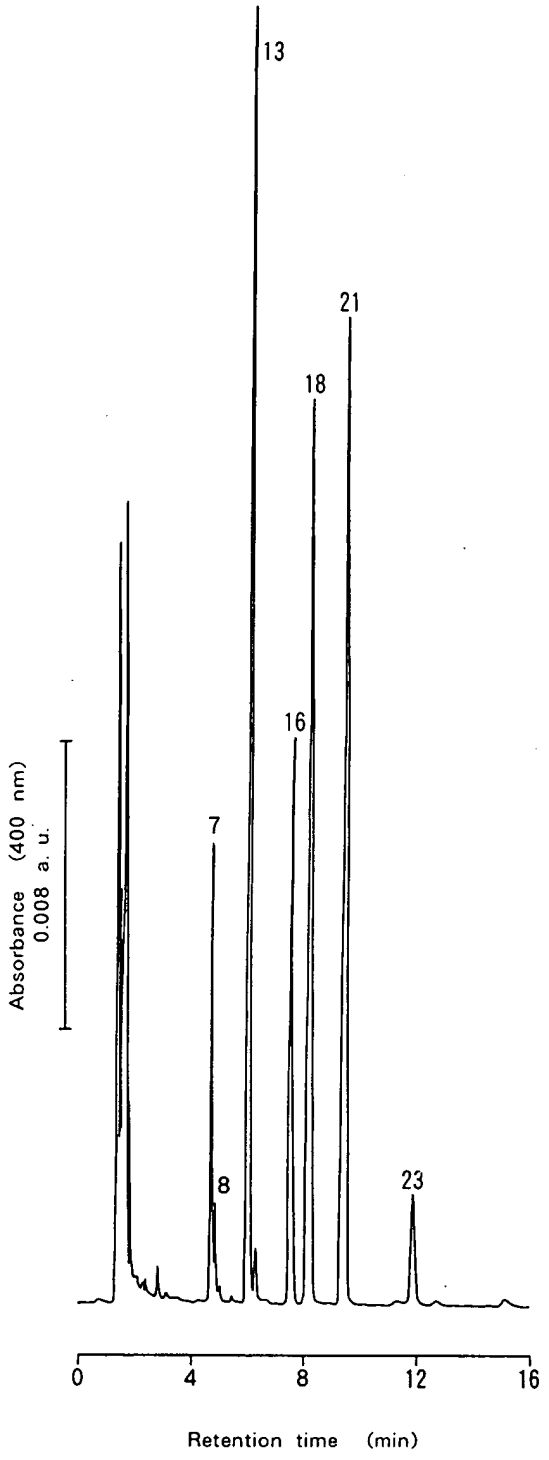

Fig. 10. Long-chain total fatty acid profile of soybeen oil. Each peak number corresponds to that in Fig. 6.

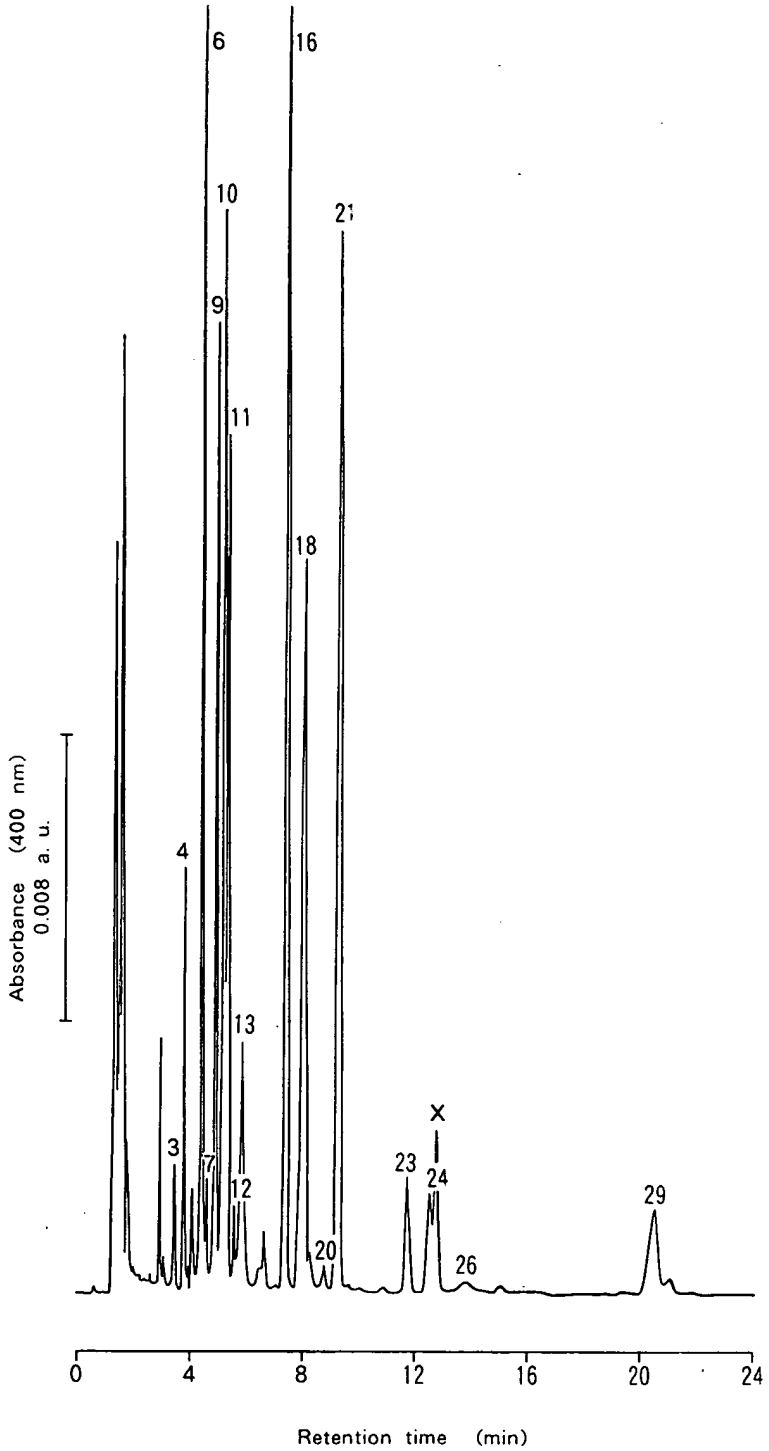

Fig. 11. Long-chain total fatty acid profile of sardine oil. Each peak number corresponds to that in Fig. 6.

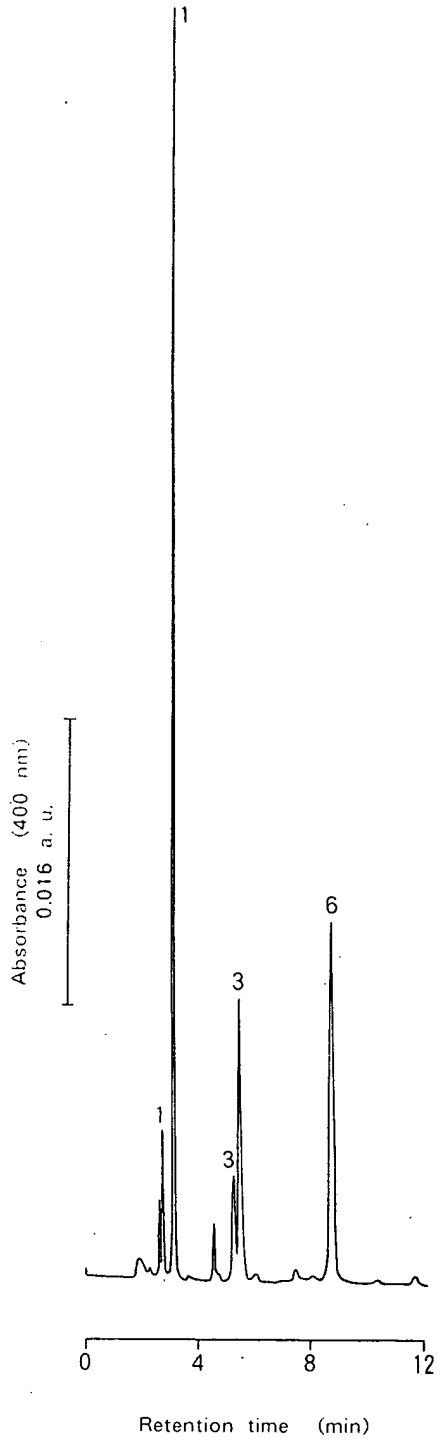

Fig. 12. Carboxylic acid profile of orange juice. Each peak number corresponds to that in Fig. 7.

5.3. Determination of mono-, poly- and hydroxycarboxylic acids in beverages

The nature and concentration of carboxylic acids, such as mono-, poly- and hydroxycarboxylic acids, in foods and beverages are of wide interest with respect to quality control. The present method was tested for the identification and determination of carboxylic acids in some beverages. The carboxylic acid profiles of typical orange juice and white wine are shown in Figs. 12 and 13, respectively. The chromatograms monitored by visible absorbance showed very clean backgrounds, and thus the carboxylic acids in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards.

The results of the determination of the major carboxylic acids in the beverages tested are listed in Table 6. According to the literature [38,41], apple and orange juices contain citric (0.25–8.47 g/l) and malic (0.20–7.20 g/l) acids. In wine, citric (0.10–3.80 g/l), tartaric (0.80–2.50 g/l), malic (0.06–3.73 g/l), succinic (0.10–0.76 g/l), lactic (0.10–4.50 g/l) and acetic (0.20–0.80 g/l) acids were found [38,40,41,75–77]. The values observed by us are compatible with those literature values.

6. Conclusions

Mono-, poly- and hydroxycarboxylic acids in foods and beverages can be directly converted into their hydrazine derivatives which absorb visible radiation. The advantage of using visible detection is that the chromatograms are simpler and more selective. This method also allows the direct derivatization of esterified carboxylic acids after saponification of the samples, and there are no work-up steps involving evaporation of solvent or aqueous washes where any carboxylic acids could be lost. The HPLC analyses described here permit the isocratic separations of those carboxylic acids in the samples with good accuracy, precision and sensitivity owing to the minimum sample preparation required. Despite its simplicity and speed the present method enables a remarkably long column life-time and therefore the method is particularly suitable for routine determinations of carboxylic acids in foods and beverages.

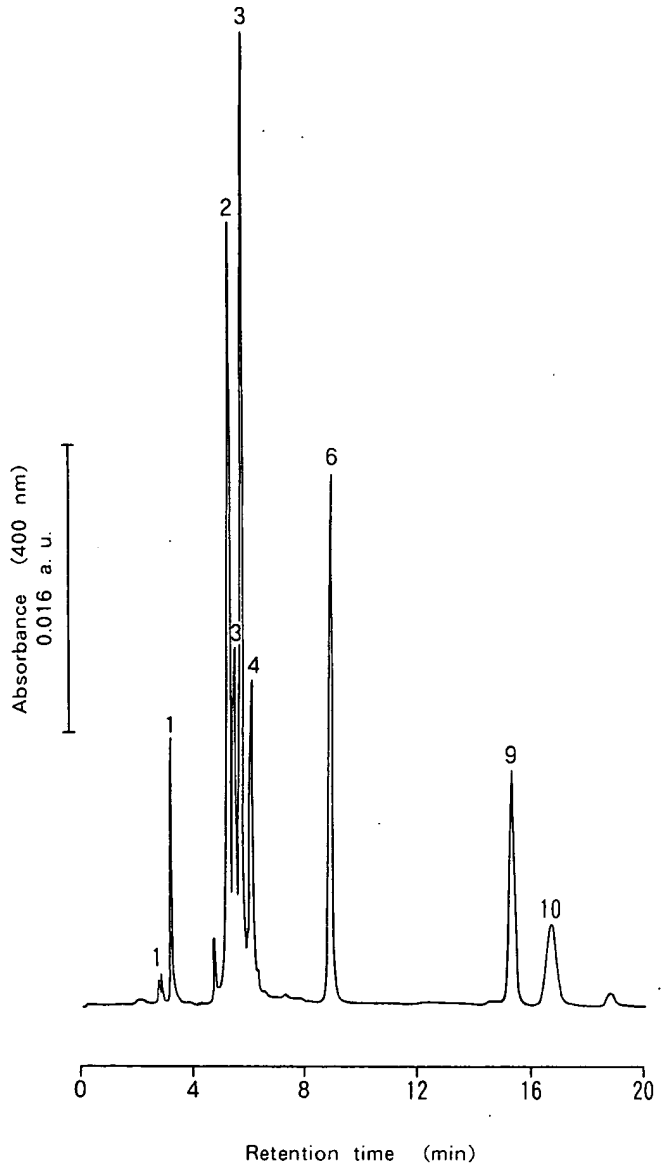

Fig. 13. Carboxylic acid profile of white wine. Each peak number corresponds to that in Fig. 7.

Table 6
Determination of major carboxylic acids in beverages by the present method[a]

Beverage	Carboxylic acid (g/l)						
	Citric	Tartaric	Malic	Succinic	L-Pyroglutaric	Lactic	Acetic
Apple juice	0.52±0.01	N.D.	2.37±0.04	N.D.	N.D.	N.D.	N.D.
Orange juice	5.35±0.14	N.D.	1.34±0.03	N.D.	N.D.	N.D.	N.D.
White wine	0.41±0.01	1.28±0.01	1.59±0.02	0.39±0.01	N.D.	0.91±0.03	0.24±0.01
Red wine	0.20±0.01	1.95±0.02	0.29±0.004	0.66±0.02	N.D.	2.99±0.05	0.58±0.01
Beer	0.16±0.01	N.D.	0.05±0.001	0.04±0.001	0.18±0.004	0.08±0.001	0.08±0.002
Japanese "sake"	0.14±0.004	N.D.	0.07±0.002	0.17±0.003	0.27±0.01	1.19±0.03	0.03±0.001

[a] Data are expressed as the mean±SD (n=3).
N.D.: Not detectable.

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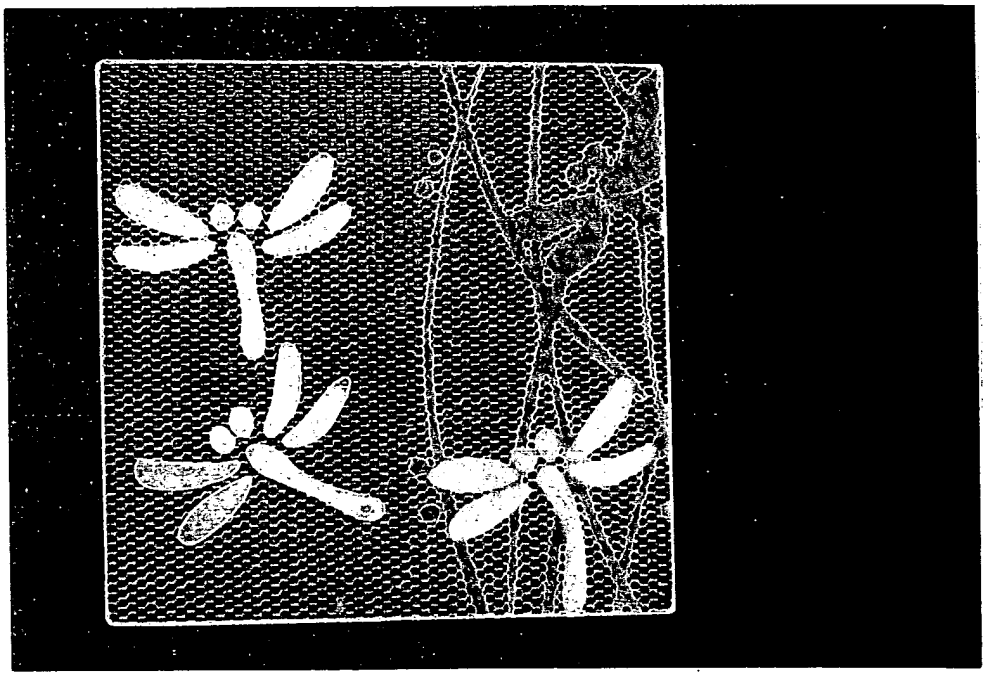
Attachment A2

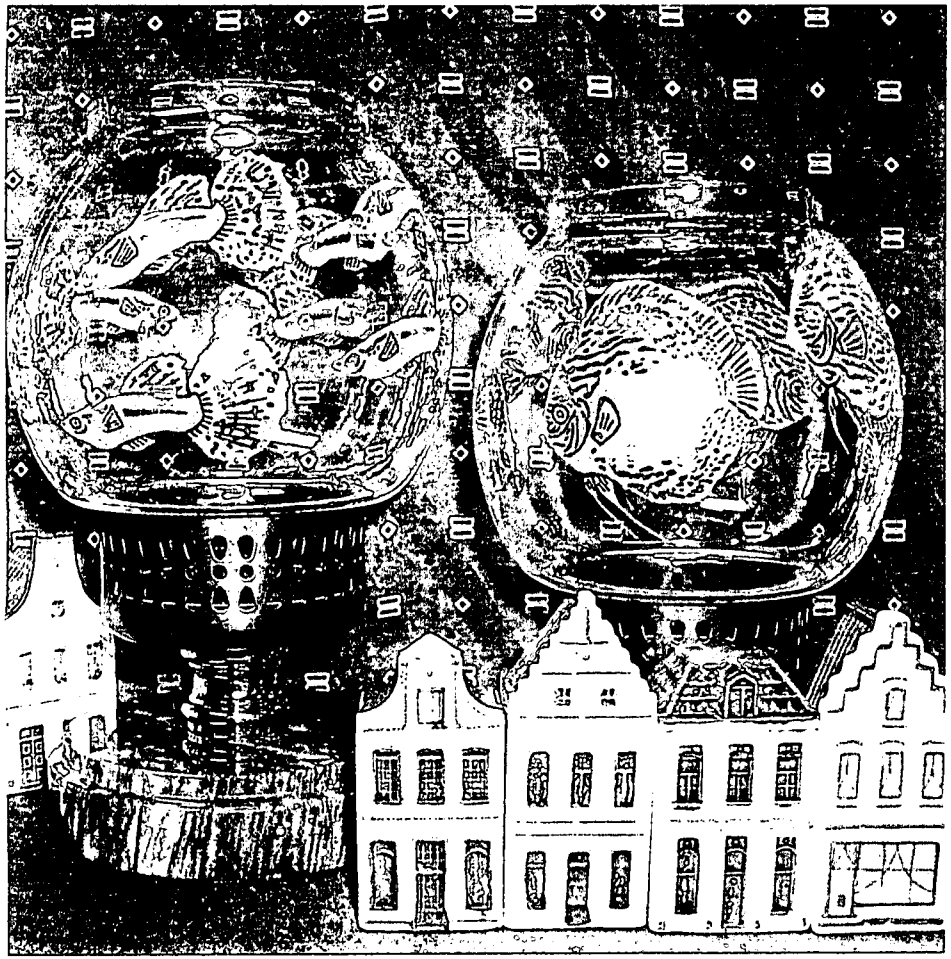
Attachment A3

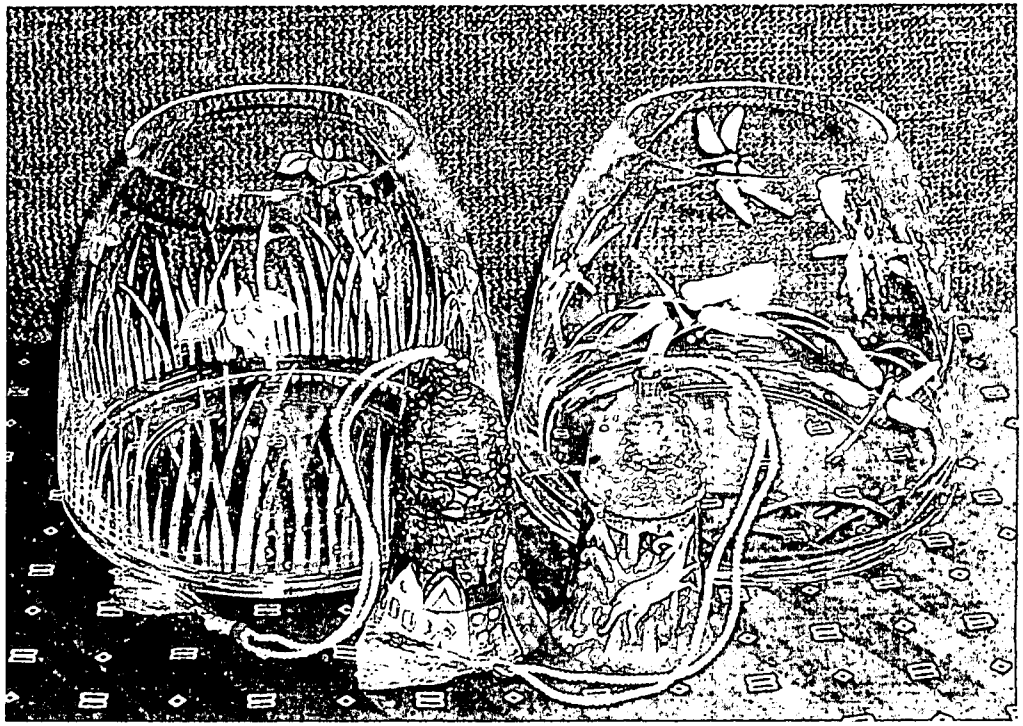


Attachment A4

Attachment B1

Attachment B2





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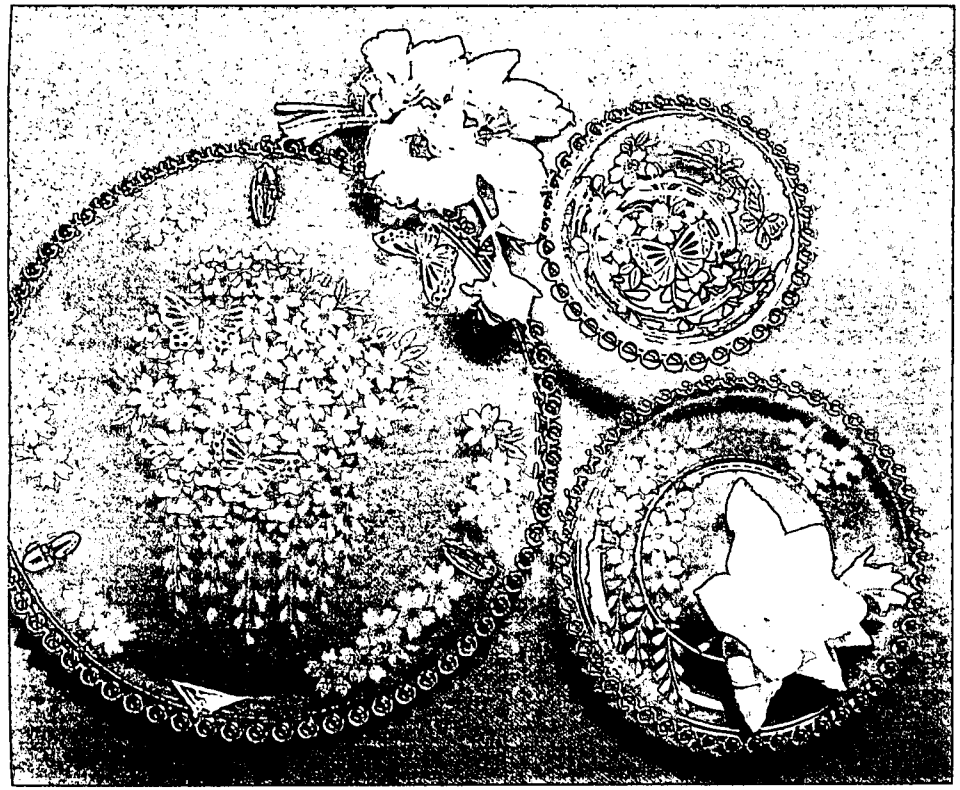

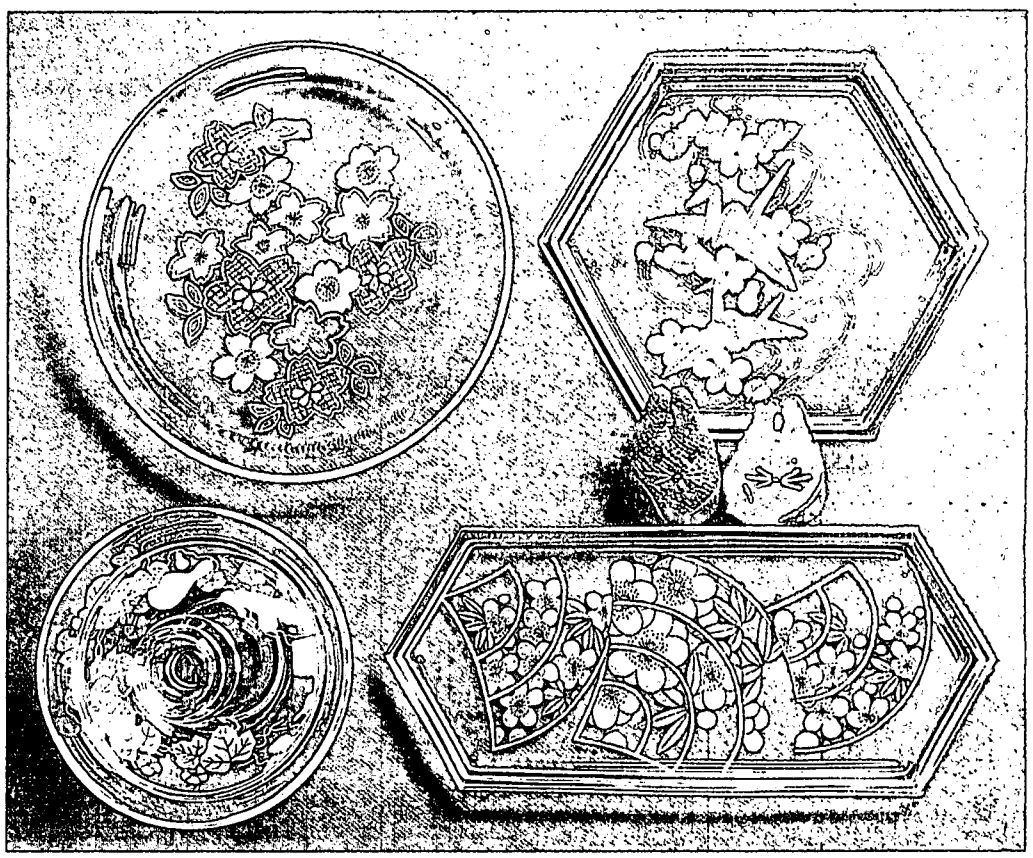

Attachment B4

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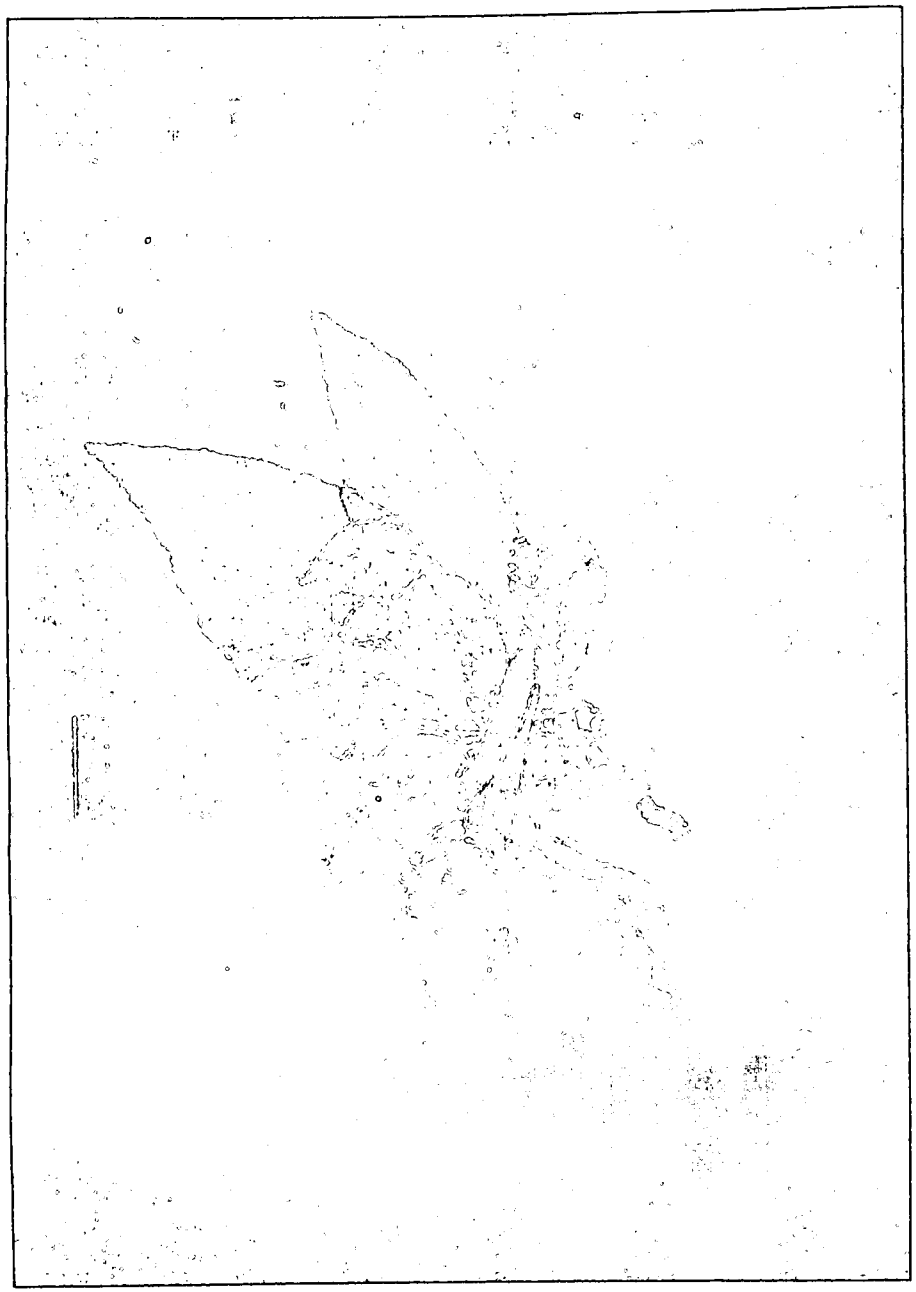

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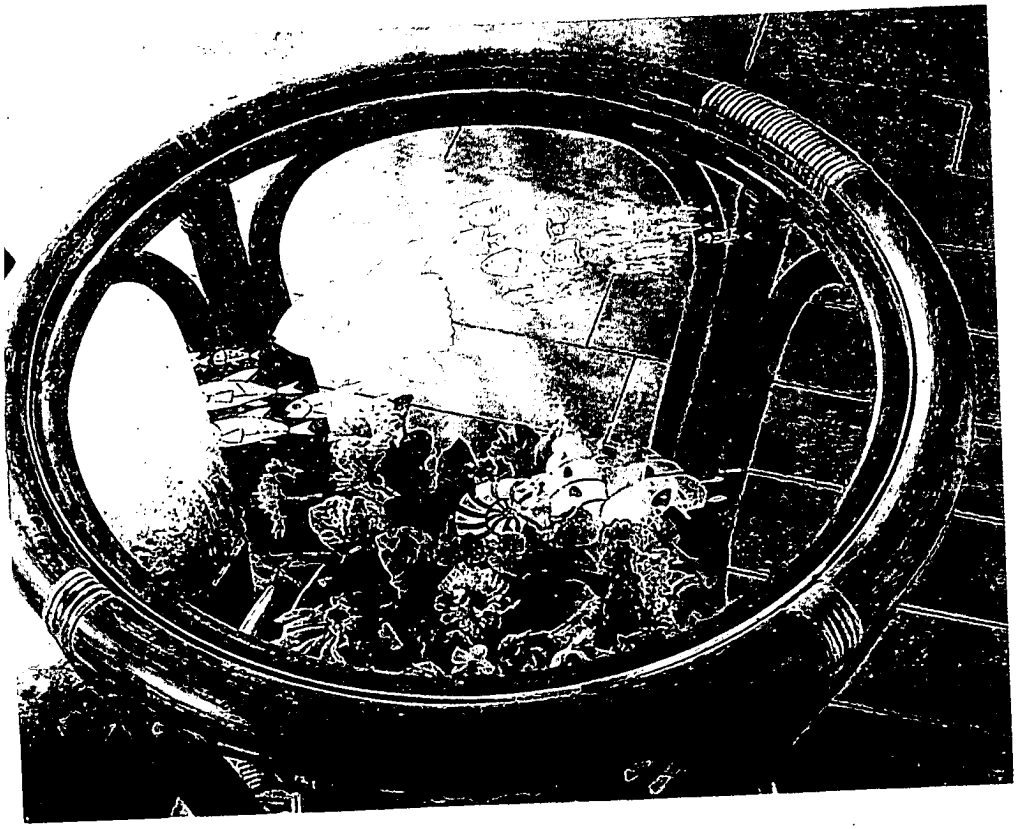

Attachment A1